

# Analytical studies on gentamicin sulphate.

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# ANALYTICAL STUDIES ON GENTAMICIN SULPHATE

Submitted by

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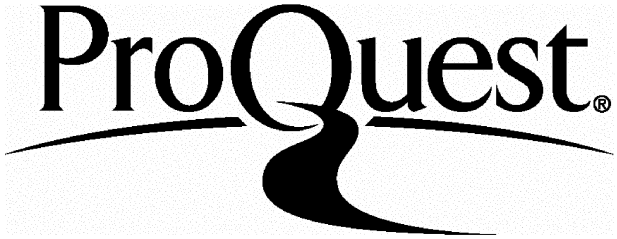
of Doctor of Philosophy

of the University of Bath

1981

This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the joint supervision of Professor R.T. Parfitt and Dr. M.G. Rowan.



Dedication

To Dr. Somkid Kraisintu, with respect and gratitude.

## SUMMARY

Pure samples of gentamicin $C_1$, $C_{1a}$, $C_2$ and seven minor components were isolated from a commercial mixture of gentamicin sulphate using column chromatography on TLC grade silica gel. A preparative HPLC method based on this separation is also described. Sufficient quantities of the minor components were isolated for spectroscopic examination. With one exception they were less biologically active against Bacillus pumilus than the 3 major components.

Quantitation of gentamicin in commercial samples by spectrofluorimetric and titrimetric methods based on the primary amino groups of gentamicin were investigated but neither proved to be suitable for routine analysis.

A high-pressure liquid chromatographic method for routine control of the composition of gentamicin in commercial samples and formulations is described. The method utilises pre-column derivatisation followed by reversed phase chromatography with fluorescence detection and uses an internal standard for quantitation. Analysis of nineteen samples of gentamicin from various sources indicates that the ratio of major components and the content of minor constituents varies with the geographical origin of the sample. The results were compared with those of a microbiological assay and the B.P. nuclear magnetic resonance (NMR) spectroscopic limit test of the same samples. The microbiological assay may be influenced by biologically active impurities whilst the NMR assay was insensitive to the presence of minor components. The method described offers a discrimating and flexible means of monitoring the composition of gentamicin.

The same HPLC method was applied to the determination of gentamicin in plasma though it was not possible to use the internal standard. The method is rapid and specific and involves no extraction of gentamicin from the plasma. It would be suitable for both routine clinical monitoring and pharmacokinetic studies.

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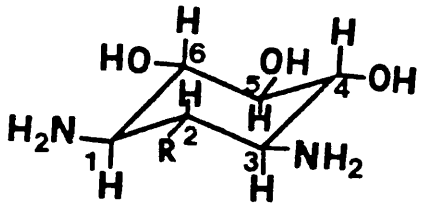
Finally, my grateful thanks to my parents, without whose indefinite patience, continuous encouragement and constant support, this thesis could never have been completed.

# CHAPTER 1

# INTRODUCTION

## Aminocyclitol antibiotics

Antibiotics may be defined as chemical substances produced by microorganisms, which have the capacity to inhibit the growth of bacteria and may be classified into a number of categories on the basis of their chemical structures. Gentamicin, the subject of this work is one of a group of carbohydrate units involved are mainly amino sugars and include an aminocyclitol ring which may be either streptamine (I) or 2-deoxystreptamine (II) (1) (Figure 1).



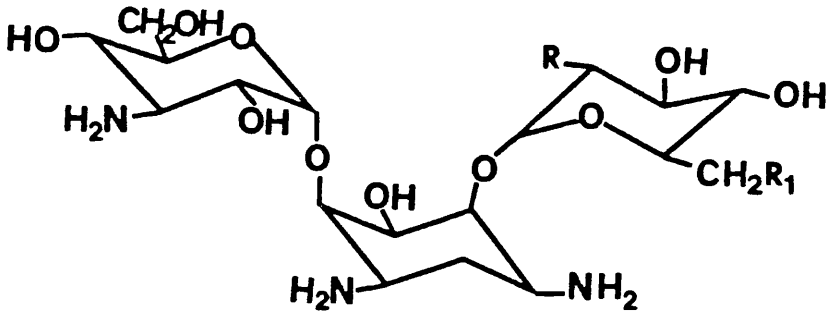

I: R = OH

II: R = H

The 2-deoxystreptamine component is readily isolated by acid hydrolysis of aminocyclitols. It is 1,3-diamino-4,5,6-trihydroxy-cyclohexane and was first described as a degradation product of neomycin A and called neamine. Subsequently it was shown to be closely related to the streptamine moiety of streptomycin which led to the use of its now more common name of 2-deoxystreptamine (2) (3).

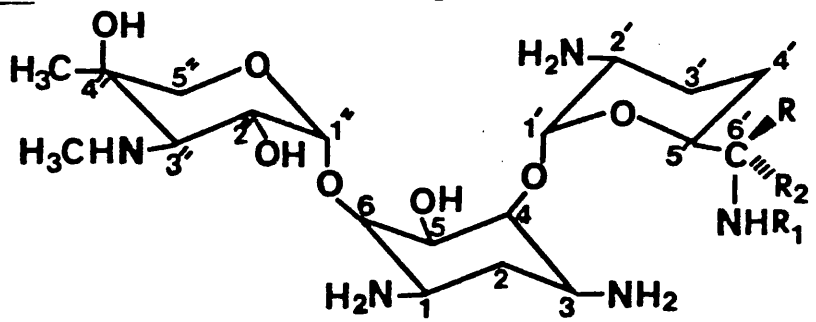
The configuration was determined by proton magnetic resonance spectroscopy and was consistent with an all-trans stereochemistry for the substituent groups on the parent cyclohexane ring (4).

A further classification of these antibiotics depends upon the placement of substituent groups on the 2-deoxystreptamine ring. Representative of compounds having 4,6-substitution are the kanamycins (Figure 2), gentamicins (Figure 3) and sisomicins (Figure 4) while those having 4,5-substitution on the 2-deoxystreptamine ring are the neomycins (Figure 5), lividomycins (Figure 6) and paromomycins (Figure 7).



| | |
|---|---|
| R = OH; $R_1$ = $NH_2$ | Kanamycin A |
| R = $R_1$ = $NH_2$ | Kanamycin B |
| R = $NH_2$; $R_1$ = OH | Kanamycin C |

Figure 2. Structure of the kanamycins



| | |
|---|---|
| R = $R_1$ = $CH_3$; $R_2$ = H | gentamicin $C_1$ |
| R = $CH_3$; $R_1$ = $R_2$ = H | gentamicin $C_2$ |
| R = $R_2$ = $R_1$ = H | gentamicin $C_{1a}$ |
| R = $R_1$ = H; $R_2$ = $CH_3$ | gentamicin $C_{2a}$ |
| R = $R_2$ = H; $R_1$ = $CH_3$ | gentamicin $C_{2b}$ |

Figure 3. Structure of the gentamicins

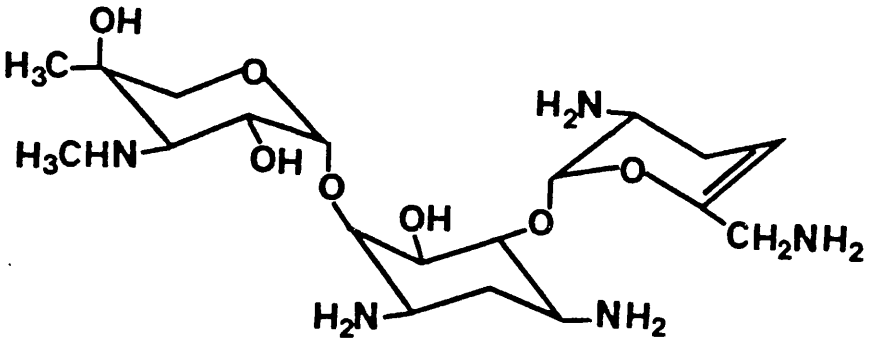
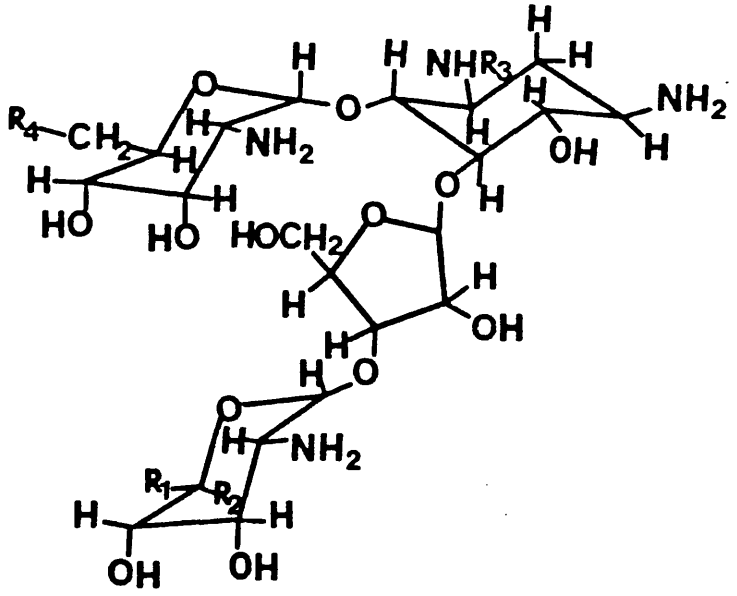


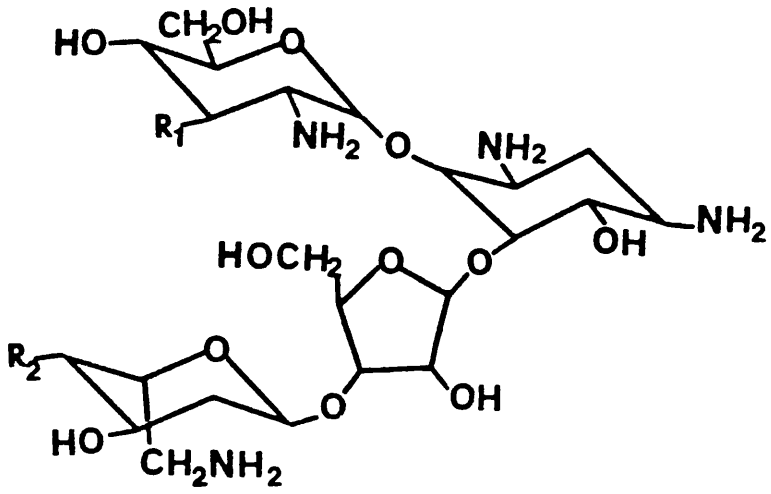
Figure 4. Structure of sisomicin.



$R_1$ = H; $R_2$ = $CH_2NH_2$; $R_3$ = H; $R_4$ = $NH_2$ Neomycin B

$R_1$ = $CH_2NH$; $R_2$ = H; $R_3$ = H; $R_4$ = $NH_2$ Neomycin C

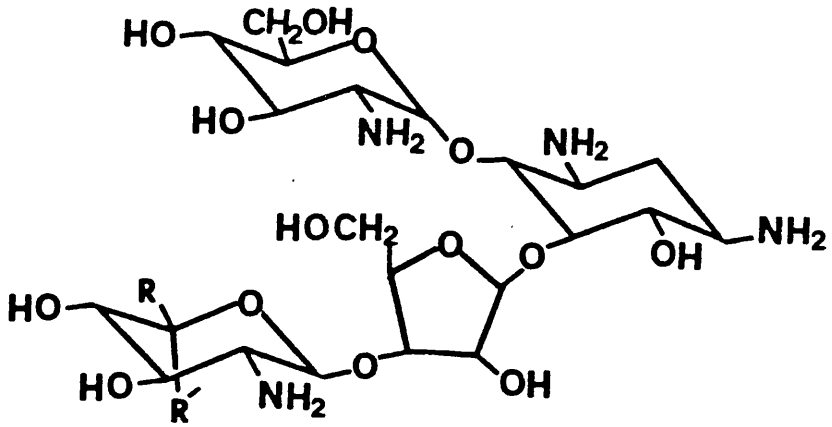
Figure 5. Structure of the neomycins.




$R_1$ = H; $R_2$ = mannosyl — Lividomycin A

$R_1$ = H; $R_2$ = OH — Lividomycin B

Figure 6. Structure of the lividomycins.




R = H; $R_1$ = $CH_2NH_2$

R = $CH_2NH_2$; R = H

Figure 7. Structure of the paromomycins.

Gentamicin

1.1 Isolation

In 1963 Weinstein and co-workers (5, 6) reported a new, basic, stable, water-soluble,broad-spectrum, antibiotic complex which was isolated from two new species of the genus Micromonospora, specifically M. purpurea sp.n. (NRRL 2953)and M. echinospora sp. n. (NRRL 2985). The taxonomy of this genus has been extensively studied by Luedemann and Brodsky (7) establishing the conformity of general features for species belonging to Micromonospora.

Isolation, purification and characterization of gentamicin was first performed by Rosselet et al. (8). Gentamicin can be readily extracted from fermentation broths by an ion-exchange procedure. The pH of the whole broth is adjusted to 2.0 with strong mineral acid to release the bulk of the antibiotic from the mycelium. After filtration of the broth, oxalic acid is added to precipitate calcium ions and the solution is neutralized with strong alkali. Calcium is separated by filtration, the filtrate passed through a column containing AmberliteIRC-50 ($Na^+$; 20 - 50 mesh) ion-exchange resin, and the spent broth is discarded.

The antibiotic mixture, containing gentamicin and co-produced antibiotics, is eluted from the resin with strong mineral acid. The neutralized eluate is then titrated with sodium dodecyl benzene sulphonate, precipitating most of the gentamicin C and leaving the co-produced antibiotics and some gentamicin C in solution. Gentamicin C dodecylbenzene sulphonate is separated by filtration, washed with water, partially dried and dissolved in methanol. The methanolic solution is passed through a column containing Amberlite IRA 401S

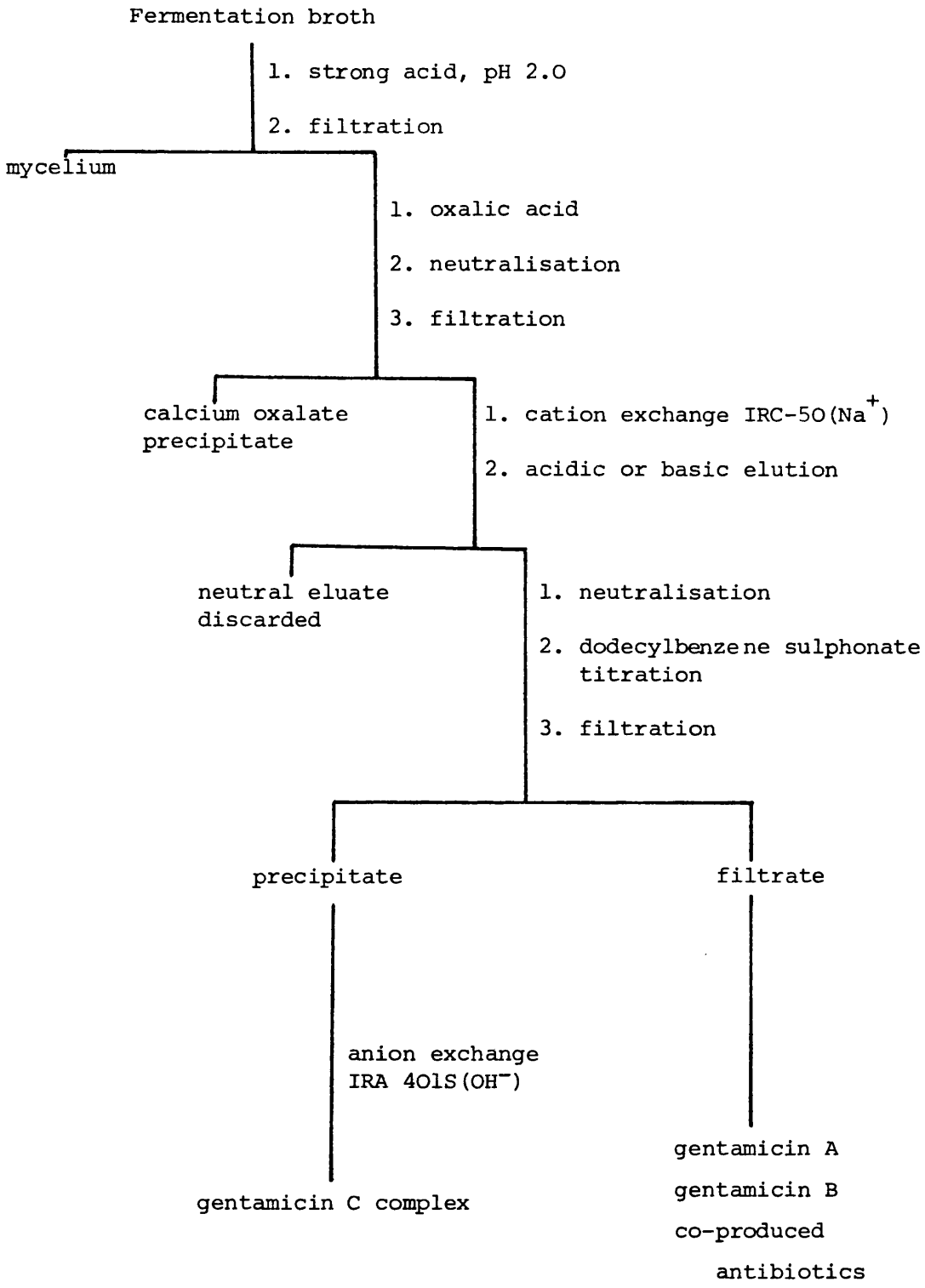



Figure 8. Isolation of gentamicin C complex from fermentation broth.

ion-exchange resin to regenerate gentamicin from its salt form. The procedure is summarised in Figure 8.

Lee and co-workers (9) have developed another method for the extraction of the complex. The pH of the whole broth is adjusted to 2.0 with sulphuric acid, stirred and filtered. The pH of the filtrate is adjusted to 7.0 with concentrated ammonium hydroxide then passed through a column of IRC-50 resin ($NH_4^+$ form). The resin is washed with water and eluted with 2N $NH_4OH$. The eluate is concentrated and air dried to give a dark brown mixture which is then passed through a column of IRA-401S resin ($OH^-$ form) and eluted with water. Eluates collected between pH 8 and 12 are combined and air-dried. After decolourisation this mixture is passed through a column containing Dowex 1x2 resin ($OH^-$ form) and the gentamicin complex eluted with water.

Lee *et al.* (10) subsequently described a method for the recovery of gentamicin from fermentation broth in which a foam separation process was evaluated using a batch system. The foam separation technique is based on differences in the surface activity of different materials which may be molecular, colloidal, or macroparticulate in size. Materials with high surface activity are selectively adsorbed or attached at the surface of bubbles rising through the liquid, and are thereby concentrated and can be separated. A substance that is not surface active itself can often be made effectively surface active by using suitable surfactant agents. Species without surface activity that are separated in this manner are called colligends and the surface active agents used to separate them are called collectors. This method enables the use of mild

conditions for stability of product, high separation efficiency when the specificity is properly exploited and allows fast separation by making use of a large interfacial area. In Lee's work, the gentamicin was a colligend and sodium dodecyl sulphate the most effective collector. From the experimental results, the optimal operating conditions selected were a pH below 9.0 and a collector-to-colligend ratio of 3:1. Bubbles were generated by pumping air into the mixture at a rate of 0.5 volume air/volume liquid/minute. Under these operating conditions, the average recovery of gentamicin was 73%.

## 1.2 Biological Activity

The antibacterial activity of gentamicin has been studied by Weinstein *et al.* (11-13) and other workers (14-17). Gentamicin is active against some Gram-positive and Gram-negative bacteria, particularly species of *Pseudomonas*, *Proteus*, *Staphylococcus* and *Streptococcus*

## 1.3 Mechanism of action

Hahn and Sarre (18) showed that protein synthesis by susceptible bacteria failed within minutes of adding gentamicin to the cultures whilst biosynthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) remained relatively unaffected for some time. From these observations it has been concluded that inhibition of protein synthesis is a specific effect of gentamicin and not merely the result of a more general disturbance of the metabolic processes. This conclusion is supported by the observation that gentamicin inhibits polymerisation of phenylalanine in a ribosome polyuridine cell-free system. Gentamicin acts by binding to the 30s particle of

the ribosome with the resultant formation of an aberrant protein synthesis complex that is incapable of participating in the biosynthesis of protein (19).

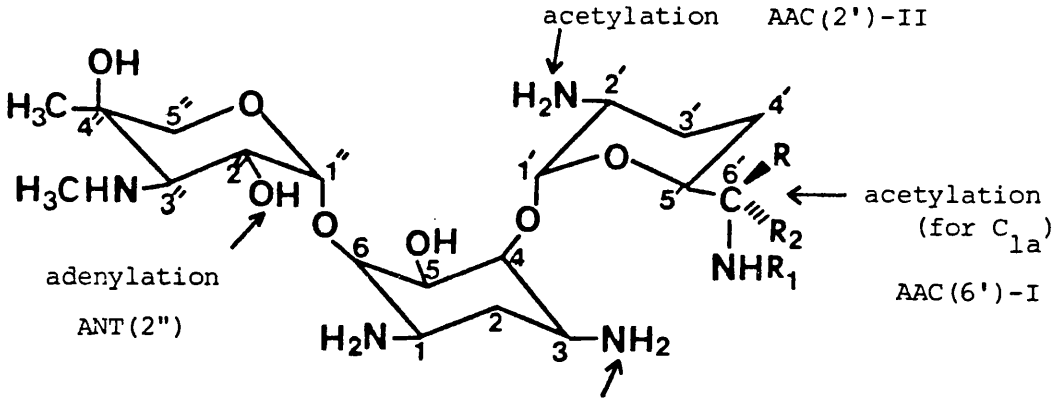
## 1.4 Resistance

Resistance to the aminoglycoside antibiotics is known and can be due either to ribosomal resistance or to the presence of aminoglycoside-inactivating enzymes.When bacteria are successively cultured in a medium containing an aminoglycoside, resistant clones are readily obtained in which the ribosomes are no longer sensitive to aminoglycosides (25). Other gentamicin resistant organisms have been shown to possess enzymes that chemically modify the molecule *via* acetylation or adenylylation (Figure 9) to produce inactive derivatives (20-26).

Enzymes AAC (3) - I, AAC (2') - II and ANT (2") would also confer resistance to kanamycin C, gentamicin A and sisomicin whereas enzyme AAC (6') - I would not. Conversely certain kanamycin resistant organisms produce a 3'-hydroxy phosphorylating enzyme and thus are susceptible to gentamicin which lacks this hydroxy group (27-29).

## 1.5 Clinical use

Clinical studies by Rabinovich *et al.*(30), Sweeder *et al.* (31), Graber *et al.* (32)and Jao and Jackson (33) confirmed its value as a broad-spectrum antibiotic especially in cases of urinary tract infection due to *Pseudomonas aeruginosa* and the *Klebsiella aerobacter* group, and secondary infections following severe burn trauma.




Gentamicin $C_1$, R = R' = $CH_3$

Gentamicin $C_2$, R = $CH_3$; R' = H

Gentamicin $C_{1a}$, R = R' = H

AAC (3) - I = Aminoglycoside-3-N-acetyl-transferase - I

AAC(2') - II = Aminoglycoside-2'-N-acetyl-transferase - II

AAC(6') - I = Aminoglycoside-6'-N-acetyl-transferase - I

ANT(2") = Aminoglycoside-2"-O-nucleotidyltransferase.

Figure 9. The enzymatic inactivation mechanisms.

## 1.6 Therapeutic levels

Peak gentamicin levels of 4 µg to 8 µg/ml of blood are generally accepted as being adequate for treating serious Gram-negative bacillary infection (34-42). Trough levels of 2 µg/ml or greater and peak levels of 12 µg/ml or greater during the administration of a normal dose of gentamicin have been associated with an increased risk of toxicity (43,44).

## 1.7 Distribution in the Tissues

Gentamicin is poorly absorbed via the intestinal tract (45) but readily absorbed on intramuscular injection and also via otic administration (11) in an as yet unknown manner. Studies with radioactive gentamicin showed that in blood 10% was associated with erythrocytes, 30% with plasma protein and 60% in solution (46-49). Gentamicin diffuses readily into thoracic duct lymph but not into the cerebrospinal fluid of most patients with uninflamed meninges (50). Gentamicin is excreted almost exclusively through the kidneys in patients with normal renal function, and high levels of the unchanged drug appear in the urine. Thus excretion is delayed where renal function is impaired, leading to an increase in the serum half-life to more than its normal value of about 120 minutes (51-57).

## 1.8 Toxicity

The three major toxic effects associated with gentamicin are ototoxicity (58-62), nephrotoxicity (63-70) and neuromuscular blockade (71). The principal toxic effect of gentamicin on the inner ear is a loss of vestibular function caused by damage to the hair cells (72). Jackson and Arcieri (43) have produced an

exhaustive review of the cases of gentamicin ototoxicity. They found that a majority of cases showed vestibular damage alone and a smaller proportion of the patients showed auditory damage alone, with only small numbers having both types of toxicity. More than half the cases of vestibular ototoxicity recovered, and in about half the instances of auditory ototoxicity damage was mild and restricted to the loss of high tones. Early detection of the signs of ototoxicity and discontinuation of the drug are essential to prevent irreversible damage (58). Nephrotoxicity caused by gentamicin appears as an acute necrosis of the proximal tubular cells of the kidney (73-77). Renal damage is usually reversible if the drug is discontinued at the first sign of renal dysfunction (78). Experiments with animals have shown that gentamicin has the ability to potentiate neuromuscular blockade where neuromuscular blocking drugs have been used (79).

## 1.9 Dosage Forms

Gentamicin is currently available for topical use and parenteral injection. Topically, gentamicin is used in a 0.1% ointment or a 0.1% cream and is effective against primary and secondary bacterial infections. Parenterally, the drug is used against susceptible Gram-negative infections. It is effective in infected surgical wounds, severe soft tissue, respiratory and urinary tract infection (80).

The usual dose of gentamicin given by intramuscular injection is 400 to 800 µg per kg body weight every 8 hours. In severe infections up to 5000 µg per kg may be given daily in divided doses. The usual dose for children is 3000 to 5000 µg per kg every 24 hours

in divided doses; infants and neonates may be given higher doses of 6000 to 7500 µg per kg daily in divided doses (81-92). The course of treatment is generally limited to 7 days.

Gentamicin has been given intravenously (43) in the same doses that are used intramuscularly. It has sometimes been given by mouth for enteric infections and to suppress intestinal flora. In the treatment of meningitis it has been administered intrathecally or intraventricularly in doses of 1000 or 2000 µg daily in conjunction with intramuscular therapy. Doses of 10,000 or 20,000µg have been given by subconjunctival infection (93,94).

1.10 Chemical description and structure

The isolation and preliminary chemical studies (8) demonstrated that gentamicin is a complex of aminoglycoside antibiotics containing the aminocyclital 2-deoxystreptamine and two additional amino sugars. The complex is isolated as an optically active amorphous powder. It is readily soluble in water, pyridine and dimethylformamide, moderately soluble in methanol, acetone and ethanol, and insoluble in diethyl ether, benzene and halogenated hydrocarbons.

Chromatographic separation of the gentamicin complex shows it to consist of 3 major components designated as $C_1$, $C_2$ and $C_{1a}$ (95). The structures of these major components have been elucidated by a combination of spectroscopic and chemical methods.

None of the gentamicin C components exhibits absorption in the visible or ultraviolet regions of the spectrum. Infrared spectra

are identical for all and, in general, are indicative of their polyamino nature (96).

Mass spectrometry gave $M^+$ peaks at m/e 477, 463 and 449 for gentamicin $C_1$, $C_2$ and $C_{1a}$ respectively corresponding to molecular formulae of $C_{21}H_{43}N_5O_7$, $C_{20}H_{41}N_5O_7$ and $C_{19}H_{39}N_5O_7$ (97). The difference of 14 mass units suggests that the three compounds differ in their degree of methylation. This was confirmed by NMR studies which showed that all three components contained a tertiary C-methyl group and an N-methyl group, but that gentamicin $C_2$ had an extra secondary C-methyl whilst gentamicin $C_1$ had both this and an additional N-methyl.

Acid hydrolysis of gentamicin C yielded 2-deoxystreptamine (Figure 1) (98). Methanolysis (98,99) produced an unusual amino-monosaccharide termed methyl garosaminide (Figure 10) and three different pseudodisaccharides, the gentamine (Figure 11).

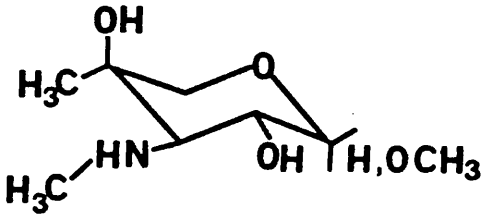
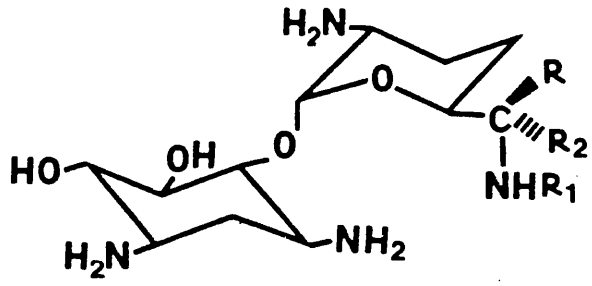



Figure 10. Structure of methyl garosaminide




purpurasamine

2-deoxystreptamine

Figure 11. Structure of gentamine C

| | |
|---|---|
| $R = R_1 = CH_3$; $R_2 = H$ | gentamine $C_1$ |
| $R = CH_3$; $R_1 = R_2 = H$ | gentamine $C_2$ |
| $R = R_1 = R_2 = H$ | gentamine $C_{1a}$ |

Garosamine was shown by chemical means and high resolution NMR (100-102) to be 3-deoxy-4-C methyl-3-methylamino-L-arabinopyranose (Figure 12).

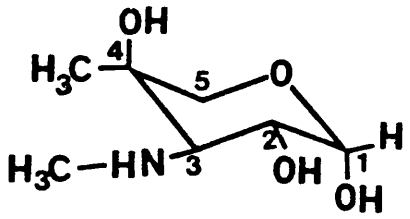
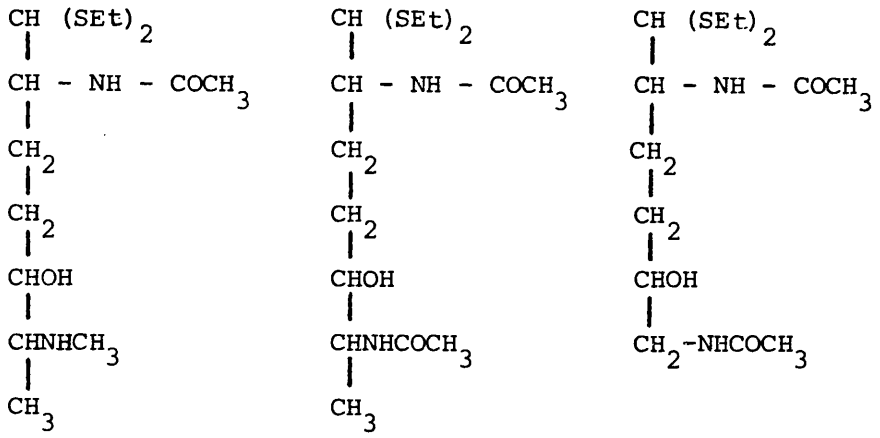



Figure 12. Structure of garosamine.

Per-N-acetyl gentamines were subjected to mercaptolysis to yield N,N'-diacetyl-2-deoxystreptamine and three crystalline diethyl-dithioacetal derivatives of a group of new aminosugars known as the purpurosamines (98). These were identified by NMR as 2,3,4,6-tetra-deoxy-2,6-diamino sugars with the structures shown in Figure 13.



(a) (b) (c)

Their structures are represented by Figures a, b and c for the compounds derived from gentamicin $C_1$, $C_2$ and $C_{1a}$ respectively.

Periodate oxidation (98) and NMR spectroscopy established the position and linkage of purpurosamine and garosamine to 2-deoxystreptamine Thus the structures of gentamicin $C_1$, $C_2$ and $C_{1a}$ were deduced to be as in Figure 3.

Recently a $^{13}C$ NMR study of a number of aminoglycoside antibiotics and their hydrolysis fragments has confirmed these structures (103).

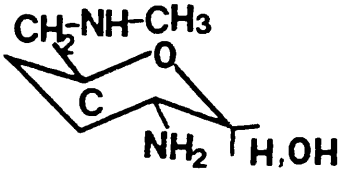
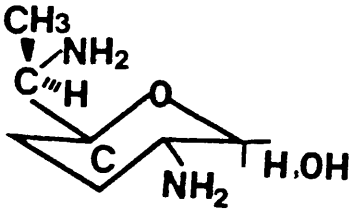
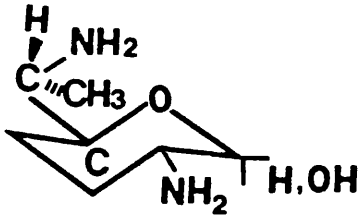
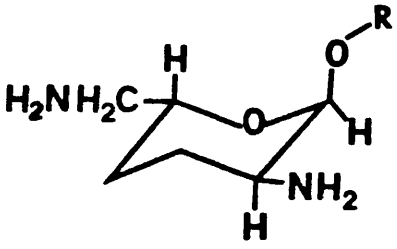
Optical rotations were performed by Kershner (104) who found that each of the gentamicin C components is dextrorotary. The same author also suggested that the gentamicin C complex consists of six compounds rather than three. These are designated, in order of separation by counter-current distribution, in a chloroform: methanol:17% ammonium hydroxide system, as $C_1$, $C_{2a}$, $C_{2-I}$, $C_{2-II}$

$C_{2\text{-III}}$ and $C_{1a}$. Thin-layer chromatography showed that $C_{2\text{-I}}$ and $C_{2\text{-II}}$ migrate identically as do $C_{2\text{-III}}$ and $C_{1a}$. Structural studies indicated gentamicin $C_{2a}$ to be an isomer of gentamicin $C_2$ with a C-6 methylaminopurpurosamine rather than a C-6 angular C-methyl group. The structures of $C_{2\text{-I}}$ and $C_{2\text{-II}}$ have been proposed to be diastereoisomeric about the asymmetric C-6 carbon of purpurosamine.

Kershner (104) also proposed that gentamicin $C_{2\text{-III}}$ differed from gentamicin $C_{1a}$ in having the L-manno configuration of the purpurosamine as opposed to the D-gluco configuration of all the other gentamicin C components.

The nomenclature used by Kershner (104) differs from that used by other authors as shown in Table 1. In this thesis the nomenclature of Daniel *et al.* (105,106) will be used.

In common with some other aminoglycoside antibiotics, the gentamicins are able to form chelates with divalent transition metal cations, notably copper (II), cobalt (II), nickel (II), manganese (II) and magnesium. Structural studies indicate a complex arrangement of metal to gentamicin possibly as an extended lattice of gentamicin molecules and metal atoms.

| Compound | Author, reference | |
|---|---|---|
| | Kershner (104) | Daniel et al (105,106)<br>Anhalt (107)<br>Weinstein (1) |
| | Name | Name |
|  | Gentamicin $C_{2a}$ | Gentamicin $C_{2b}$ |
|  | Gentamicin $C_{2-I}$ | Gentamicin $C_2$ |
|  | Gentamicin $C_{2-II}$ | Gentamicin $C_{2a}$ |
|  | Gentamicin $C_{2-III}$ | |

## 1.11 Assay of Gentamicin

### 1.11.1 Microbiological assay

The basic procedure for most microbiological assays of gentamicin is an agar-diffusion method. This involves the use of a suitable agar medium carrying a test organism that is sensitive to the antibiotic to be assayed. Standard solutions of antibiotics and unknown samples are placed in wells punched into the agar plate. After incubation for 12 to 48 hr, the zones of growth inhibition of the test organism around the wells are measured and compared with the standards to determine the antibiotic potency of the unknown samples.

Microbiological assays in general provide versatility and simplicity and are relatively inexpensive, but have several disadvantages. Firstly, the time required for conventional microbiological assays is 12 - 48 hours, although microbiological assays with shorter incubations (4-6 hr) have been described for gentamicin (108-110). Secondly, the basic procedure is not specific and thus will not allow an accurate assay of gentamicin in the presence of other antibiotics. This problem arises in assying the serum levels in patients who are receiving other antibiotics in additional to an aminoglycoside. This interference can be circumvented by removing the interfering antibiotics, by adding a substance to the sample or medium that will inactivate the potentially interfering agent(s), or by using a test organism that is susceptible only to the aminoglycoside.

Oden *et al.* (111) described two microbiological assays for

gentamicin. A standard curve disc-plate assay with Staphyloccus aureus ATCC 6538P was used for the analysis of raw materials. The method used for serum determinations was a standard curve cylinder-plate assay utilizing the more sensitive Bacillus subtilis ATCC 6633 as the test organism.

Carlström et al. (112) used electrophoresis in agarose gel to identify and quantify gentamicin in serum in the presence of carbenicillin, rifampin and clindamycin. After the drugs had been separated by electrophoresis, indicator bacteria in a thin agar layer were overlaid and the zones of growth inhibition determined.

Sabath et al. (113) reported a determination of gentamicin in 0.08 ml samples of serum or plasma with a modification of the standard agar diffusion method. Gentamicin was assayed, in the presence of penicillins or cephalosporins by inactivating them with a zinc-requiring"broad-spectrum" β-lactamase. The use of multiple-antibiotic-resistant strains also allows determination of gentamicin in the presence of other commonly used antibiotics. Alcid and Seligman (114) determined gentamicin concentration using a multiple-antibiotic-resistant strain of Staphylococcus epidermidis in an agar well diffusion assay.

Rapid gentamicin bioassays have been described by Warren et al. (115) using Staphylococcus aureus ATCC 6538 P and by Lund et al. (116) using a multiple-antibiotic-resistant strain of Klebsiella pneumoniae. The results were obtained in 2 - 4 hr.

In addition to these methods which directly measure bacterial inhibition a number of assays have been based on other consequences of the antibiotic's effect on bacteria.

Urban (117) described a rapid micromethod for gentamicin assay in serum by utilization of bacterial reduction of nitroblue tetrazolium. The reduction of colourless nitroblue tetrazalium (NBT) to dark blue formazan by Escherichia coli is diminished by pre-incubation with antibiotics. The method has been modified to allow assays on as little as 50 µl of serum containing 0.2 - 5.5 mg/l of gentamicin.

Rapid bioassays of gentamicin in clinical serum specimens, based on determination of adenosine triphosphate (ATP) levels in bacterial cultures exposed to gentamicin, have recently been developed. Harber and Asscher (118) determined the total ATP level in bacterial cultures, and Nilsson et al. (119) selectively determined the intracellular ATP levels. Both methods require extraction of intracellular ATP. Nilsson (120) described the relationship between intracellular and extracellular ATP levels in Escherichia coli cultures exposed to various concentrations of gentamicin. The bioassay compared serum specimens with standard solutions of gentamicin.

Jacob et al. (121) and Noone et al. (122-125) described the use of a method based on the monitoring of the pH change brought about by the urease activity of Proteus mirabilis. Proteus species can split urea enzymatically with the production of ammonia and a consequent rise in pH. The presence of antibiotics reduces this

activity and a less rapid rise is observed. Since gentamicin is most active in alkaline solution, it seemed possible that a sensitive assay method could be devised based on this effect.

Another method which depends on pH measurements has been described by Faine and Knight (126). The method achieved an accuracy and reproducibility comparable with plate methods but took only 90 mins to complete. The principle is based on the measurement the fall in pH in a culture containing a heavy inoculum of Klebsiella aerogenes. Fermentation is inhibited by antibiotic thus slowing the fall of pH. The rate of the pH change is related to the concentration of antibiotic.

1.11.2 Enzymic Assay

Daigneault et al. (127) presented a detailed account of various enzymes which transfer adenyl, acetyl and phosphoryl groups to various positions of the three six-membered rings of the amino-glycosides. These enzymes are often present in organisms that carry resistant (R) factors and are believed to be responsible for the inactivation of gentamicin. These enzymic reactions form the basis of several radioenzymatic assays which have been developed for gentamicin (128-133).The principle of this assay depends on the specific enzymatic transfer of a radioactive modifying group to gentamicin. Different workers have employed different enzymes. Thus Smith et al.(128) used enzyme isolated from a mutant of Escherichia coli which transfers adenyl groups from ATP to the 2" position of gentamicin. Adenylated gentamicin, but not ATP, is positively charged and therefore binds to a negative-charged phosphocellulose ion exchange paper. When aliquots of a reaction mixture, containing

$^{14}$C-ATP, are pipetted onto phosphocellulose paper and washed to eliminate non specifically bound radioactivity, the phosphocellulose bound radioactivity is a measure of the adenylated antibiotic.

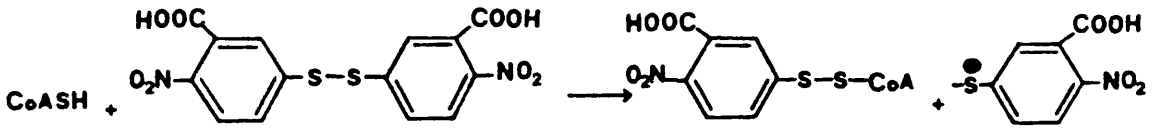
Shannon and Phillips (134) described an assay for gentamicin in serum, plasma and urine which used $[1\text{-}^{14}C]$ acetyl coenzyme A and aminoglycoside 2'-N-acetyltransferase, an enzyme which acetylates all 3 major components of gentamicin. The separation of acetylated gentamicin from acetyl donor was again achieved by the use of phosphocellulose paper.

6'-N-acetyl transferase is less advantageous since it does not detect gentamicin $C_1$ which lacks a primary 6' amino group.

Williams *et al.* (137) described a spectrophotometric assay for gentamicin in blood serum. The assay used a purified enzyme from *Escherichia coli* JR 88/C600 which catalyzes the acetylation of gentamicin with the production of coenzyme A. CoA production is linked to a chemical reaction with a sulphydryl reagent to produce stoichiometric amounts of a sensitive chromophore which can be monitored in the visible spectrum.

gentamicin + acetyl CoA $\longrightarrow$ acetyl-gentamicin + CoASH (1)

Sulphydryl groups rapidly react with 5,5'-dithiobis-(2,2) nitrobenzoic acid to produce a stoichiometric amount of a disulphide and thionitrobenzoic acid as shown in reaction (2)




(2)

Thionitrobenzoic acid production was monitored by recording the increase in absorbance at 412 nm. The components of reaction (2) do not affect the progress of reaction (1).

Enzymatic assays have the advantage of simplicity, accuracy and reproducibility and correlate well with both microbiological and radioimmunoassays (135). They are about as sensitive as microbiological assays and can be completed in 2 to 3 hr. A particular disadvantage when applied to gentamicin in serum is that the presence of carbenicillin gives low values perhaps due to the formation of a gentamicin-carbenicillin adduct (136).

1.11.3 Immunoassays of gentamicin

Immunoassays depend upon the ability of specific antibodies to bind to the antigens which were used to stimulate their production. If labelled and unlabelled antigens are mixed with the appropriate antibody they compete for binding sites, the proportion of the labelled antigen bound reflecting its abundance in the mixture. Thus if calibration curves are prepared using known amounts of unlabelled antigen and antibody it is possible to estimate the amount of antigen in an unknown sample. Free and bound drugs may be separated by various methods such as electrophoresis, solid-phase adsorption, or chromatography (138-141). If the antibodies used were raised in e g. rabbit separation may be achieved by adding anti-rabbit gamma globulin to precipitate the antigen and antibody complex (double antibody method). In the double-antibody method the reaction mixture is centrifuged and the radioactivity in the precipitate is assayed.

A major advantage of the radioimmunoassay method is its high sensitivity (micrograms per litre of serum). If standards are prepared in advance, the assay can be performed in about 2 hr. The lack of a radioactive measurement facilities in many clinical laboratories, the necessity of handling radioactive materials, and its relatively high cost are major disadvantages of radioimmunoassay. Also essential to radioimmunoassay are a purified form of antigen for radiolabelling and also antibodies to the antigen. Because aminoglycoside antibiotics are not highly immunogenic, these haptens must be conjugated to a carrier protein, for specific antibodies to be raised.

Ashby et al. (142) described a single radioimmunoassay that can measure gentamicin, tobramycin and amikacin in the same sample, concurrently. A single reagent system composed of a pool of three very specific antisera was used in the procedure. The antisera combination did not alter the specificity of the procedure. Different dilutions of antisera to these drugs are combined to give 45 - 55% initial binding of the $^{125}$I-labelled antibiotics. Iodinated derivatives of these three antibiotics were prepared by a modified Bolton and Hunter procedure (143). This assay measures all three aminoglycosides with a single assay system. Recovery and precision were excellent, but a longer time was needed to optimize the whole system. The short shelf-life of $^{125}$I-labelled antibiotics may also be a problem.

Shaw et al. (144) described a determination of serum gentamicin by a non-isotopic immunoassay. The method is based on partial

quenching of fluorescence observed when fluorescein-labelled gentamicin is bound by anti-gentamicin serum. The fluorescence intensity of the labelled gentamicin in an unseparated immunoassay incubation mixture therefore serves to indicate the extent of binding, which is related to the amount of competing unlabelled gentamicin The method is termed "quenching fluoroimmunoassay".

Wason et al. (145) reported a polarisation fluoroimmunoassay for gentamicin based on detection of the extent of antibody binding of fluorescein-labelled gentamicin by measuring fluorescence polarisation. The principle is that gentamicin-fluorescein isothiocyanate-gentamicin; antibody complexes scatter incident polarized light to a much greater degree than do the small molecules of gentamicin-fluorescein isothiocyanate, and antibody binding can be assessed by changes in fluorescent intensity.

Burd et al. (146) reported a homogenous reactant-labelled fluorescent immunoassay for the measurement of therapeutic gentamicin concentrations in human serum. A derivative of umbelliferyl-β-galactoside was coupled covalently to the drug and this conjugate was found to be non fluorescent under assay conditions but fluorescent umbelliferol could be released by the action of β-galactosidase. When the drug/dye conjugate was bound to anti-gentamicin antiboy it was inactivated as an enzyme substrate. This inactivation was relieved by the presence of gentamicin in competitive binding reactions. Hence, the rate of production of fluorescence was proportional to the gentamicin concentration. This assay required only 1 µl of serum.

Standefer and Saunders (147) described a technique in which a peroxidase-gentamicin conjugate was mixed with a gentamicin standard

or serum together with an immobilised gentamicin antibody. The extent of the binding of conjugate to the antibody was observed by adding a chromogenic substrate for the peroxidase reaction. The absorbance of the product was inversely proportional to the amount of gentamicin in the reaction well. This assay can be completed in 30 minutes.

A haemagglutination inhibition assay for gentamicin has been described by Mahon et al. (148). Antigen in the form of gentamicin complexed to bovine serum albumin was covalently attached to sheep erythrocytes. When antiserum to this complex was added to a sample containing more than 10 mg of the drug per litre, haemagglutination was inhibited.

A non-equilibrium enzyme immunoassay of gentamicin in serum was described by Mattiasson et al.(149). This method utilized a thermometric enzyme-linked immunoadsorbent assay (TELISA). A microcolumn filled with a polymer support containing covalently bound antibodies and well insulated from the surroundings was used. The gentamicin sample was mixed with a known amount of gentamicin-catalase conjugate prior to passage down the column. On passage of the sample through the immunosorbent in the enzyme thermistor column, competitive binding of native and enzyme labelled antigen to the antibodies takes place. The amount of enzyme bound to the column was measured by introducing a pulse of a suitable substrate. The extent of reaction was determined by measuring the temperature change produced. The amount of enzyme bound is a function of the concentration of native antigen in the sample.

Phillips et al. (150) have compared, in a clinical laboratory,

three methods for estimating the concentration of gentamicin in serum. The adenylase method is most accurate, but requires considerable skilled technical time and expensive apparatus. The urease method requires an accurate pH meter but is otherwise inexpensive. Although it produces results most rapidly, it requires considerable technician time and is the least accurate. The agar diffusion method requires no expensive apparatus, least technician time, and produces results of acceptable accuracy. It does, however, take longer than the other two methods to produce results.

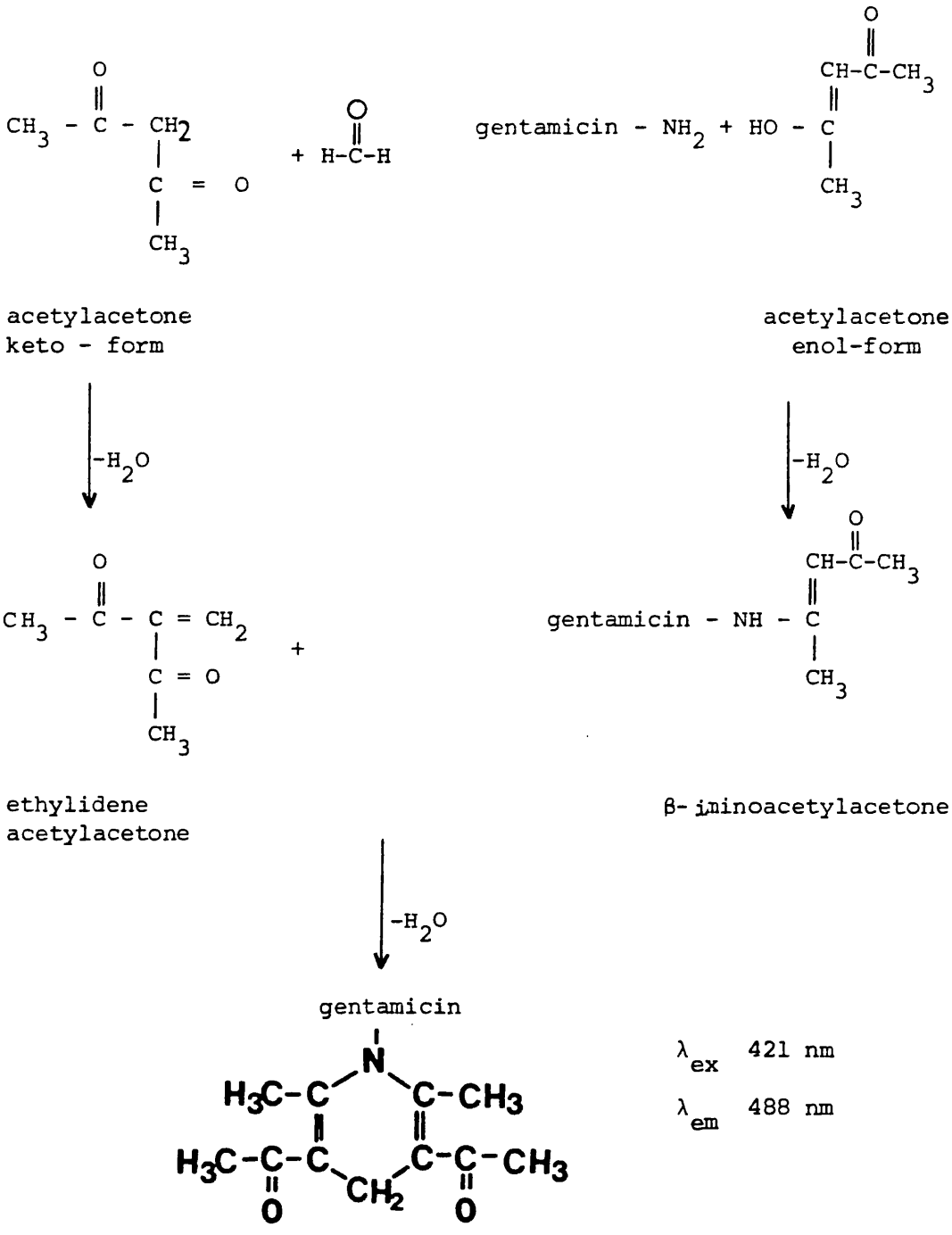
### 1.11.4 Spectrophotometric and spectrometric analysis of gentamicin

#### 1.11.4.1 Colourimetry

The reaction of gentamicin with ninhydrin has been used to determine gentamicin in pure preparations (151). Kartseva (152) has described a method based on the ability of gentamicin to form a coloured complex with copper to control the preparation and purification of commercial gentamicin.

#### 1.11.4.2 Fluorimetry

Csiba (153) presented a chemical method for the quantitative determination of gentamicin in biological fluids. Gentamicin was separated from serum and urine by ion exchange chromatography and determined spectrofluorometrically by means of a fluorescent dihydrolutidine derivative. This was formed by condensation of the primary amino groups with acetylacetone and formaldehyde, under acidic conditions (pH 2.6).




3,5-diacetyldihydro-lutidine

1.11.4.3 Proton magnetic resonance spectrometry

The proportions of the main components present in gentamicin sulphate can be monitored by $^{1}H$ nuclear magnetic resonance spectrometry (154). The method depends on the measurement of the peak height of signals due to N-methyl and C-methyl groups present in all three main components and of those present in $C_1$ and $C_2$ only. By defining peak height ratios the composition of gentamicin may be specified within acceptable limits. The purity and potency must be controlled by other means. The proton magnetic resonance method is very rapid, simple in execution and applicable both to the bulk antibiotic and to the injection formulation. However, it is capable of providing control only over the relative proportions of the gentamicin C components. It provides no control over other gentamicins nor over the proportion of gentamicin C complex in the material.

1.11.4.4 Mass spectrometry of gentamicin

This technique depends on the ionization and mass fragmentation of compounds by high voltage and the subsequent separation of the mass ions in a magnetic field. Daniels et al. (97) investigated the electron impact and isobutane chemical ionization mass-spectral fragmentation pattern of gentamicin components $C_1$, $C_2$ and $C_{1a}$. Parfitt et al. (155) used chemical ionization and field desorption mass spectrometry. According to these authors, field desorption under optimum conditions resulted in little glycosidic cleavage and the $[M+1]^+$ ions dominated in the spectra. These methods can show the component profiles in commercial gentamicin samples. Related compounds as trace impurities may be identified by their $[M + H]^+$ ions.

## 1.12 Separation of gentamicin components

Commercial gentamicin C is known to be a mixture of several related antibiotic substances. A number of methods have been developed to separate, characterise and quantify the individual components.

### 1.12.1 Paper chromatography

Table 2 shows a comparison of Rf values of the major gentamicin components $C_1$, $C_2$ and $C_{1a}$ using various mobile phases.

| Paper chromatographic system | Gentamicin components $C_1$ | $C_2$ | $C_{1a}$ | Reference |
|---|---|---|---|---|
| 80% methanol plus 3% sodium chloride (wt/vol) (1:1), descending[a] | 0.57 | 0.56 | 0.48 | 1 |
| propanol:pyridine:acetic acid: water (6:4:1:3), ascending (v/v) | 0.34 | 0.30 | 0.22 | 1 |
| 80% phenol, ascending (v/v) | 0.45 | 0.45 | 0.45 | 1 |
| chloroform:methanol:17% ammonia (2:1:1)[c] | 0.67[b] | 0.40[b] | 0.21[b] | 95<br>156 |

a = paper buffered with 0.95M $Na_2SO_4$ and 0.05 M $NaHSO_4$

b = $R_t$ = distance of zone from origin/distance from origin to end of paper at t = 6h

c = lower phase.

1.12.2 Thin-layer chromatography

Table 3 shows a comparison of various thin-layer chromatographic methods for the separation of the gentamicin complex

| Support material and solvent system | Gentamicin components $C_1$ | $C_2$ | $C_{1a}$ | Reference |
|---|---|---|---|---|
| Silica gel G, chloroform:methanol: 17% ammonia (2:1:1) | 0.74 | 0.46 | 0.27 | 95 |
| Chrom AR$^R$(Silicic acid - glass fibre sheets),chloroform: methanol: 28% ammonia (2:1:1) | 0.63 | 0.37 | 0.20 | 157 |
| Silica gel G, chloroform: methanol: conc. ammonia (1:1:1)[a] | 0.44 | 0.39 | 0.35 | 158 |

[a]These workers also detected six minor components which they designated gentamicin $X_1$, $X_2$, $X_3$, $X_4$, $X_5$ and $X_6$. Their Rf values were 0.48, 0.31, 0.27, 0.25, 0.23 and 0.18 respectively.

Table 4 shows the Rf values in various systems for some of the co-produced antibiotics (159)

| | System 1 (mm from origin) | System 2 $R_F B_1$ (a) | System 3 $R_F$ |
|---|---|---|---|
| Gentamicin $C_{1a}$ | - | - | 0.20 |
| Gentamicin $C_2$ | - | - | 0.37 |
| Gentamicin $C_1$ | - | - | 0.63 |
| Gentamicin A, [b] | 0.0 | 0.36 | 0.10 |
| Gentamicin A | 0.0 | 0.41 | 0.16 |
| Gentamicin B | 9.0 | 0.52 | 0.22 |
| Gentamicin X | 13.5 | 0.55 | 0.28 |
| Gentamicin $B_1$ | 27.0 | 1.00 | 0.40 |

a $R_F B_1 = \frac{\text{distance component from origin}}{\text{distance component } B_1 \text{ from origin}}$

b Present in component A as a trace

System No. 1. chloroform - methanol-17% ammonia (paper)

System No. 2. 2-butanol - tert - butanol - methanol - 27% ammonia (paper)

System No. 3. Chloroform - methanol - 28% ammonia (TLC)

In both TLC and paper chromatography a number of methods have been used to detect the compounds. The most usual is ninhydrin used under acidic (158) or basic (1) conditions. Alternatively a microbiological method may be used either by means of a bio-autographic technique (1, 153, 160-162), or be eluting the compounds prior to a microbiological assay (163). This latter method affords a means of quantifying the components. Quantification after

spraying with chemical reagents has also been achieved by densitometric (158, 160, 161, 164) or fluorimetric (165) scanning.

1.12.3 Column chromatography

Ion exchange chromatography has proved useful both in isolating gentamicin C from co-produced antibiotics and in separating the components of gentamicin C. Bérdy et al. (166) isolated 25 aminocyclitol antibiotics from the crude culture broth of Micromonospora purpurea var. nigrescens MNG-122 strain by means of repeated chromatography on carboxyl - or sulphonyl-type ion-exchange resins and dextran gels. Gentamicin $C_1$, $C_{1a}$, $C_2$, A, B, $B_1$, $X_2$, sisomicin, garamine, gentamine and seven other compounds were identified. Gentamicin A has also been isolated by chromatography on Dowex 1 x 2 (167). Thomas and Tappin (151) used cellulose phosphate P 11 to separate commercial gentamicin C into four major and three minor components. These all showed optical activity and gave a positive reaction with ninhydrin but only the four major components were microbiologically active.

The gentamicin C complex can be separated into 3 major components by employing cellulose or chromosorb W chromatographic columns with chloroform, methanol and 17% ammonia as the solvent system (95). Cooper et al. (102) used silica gel with the solvent system chloroform, isopropanol and concentrated ammonia to isolate the major components of gentamicin C on a preparative scale.

Wagman et al. (159) has described a similar method for the separation of the minor constituents produced in the gentamicin fermentation by Micromonospora purpurea NRRL 2953. The column used

was silica gel and the solvent system was chloroform, methanol and 28% ammonia. Compounds isolated included gentamicin A, $A_1$, B, $B_1$ and X.

1.12.4 Counter-Current -Distribution

A Craig-type counter current distribution apparatus has been used by many authors (104, 105,168) in the separation of the gentamicin complex. Except for minor variations all of the reported methods are very similar and use a solvent system composed of chloroform, methanol and 17% ammonia. In addition to gentamicin $C_1$, $C_{1a}$ and $C_2$ this method also separates gentamicins $C_{2a}$ and $C_{2b}$. These relatively minor components have been identified as the 6'-C epimer of gentamicin $C_2$ and 6'-N-methyl gentamicin $C_{1a}$ respectively. (168). The microbiological activity of both components is substantially the same as gentamicin $C_1$, $C_2$ and $C_{1a}$. The advantages of Craig distribution for the separation of the gentamicin C complex lie in its ability to handle large quantities of material and to separate the components without prior derivatization. However, the solvent system used is susceptible to large variations in saturation caused by relatively small temperature fluctuations (104). In addition, their relatively strong basicity allows for precipitation of atmospheric carbon dioxide as ammonium carbonate salts. These problems could also occur in those chromatographic systems which use a similar solvent mixture.

1.12.5 High-performance liquid chromatographic analysis of gentamicin

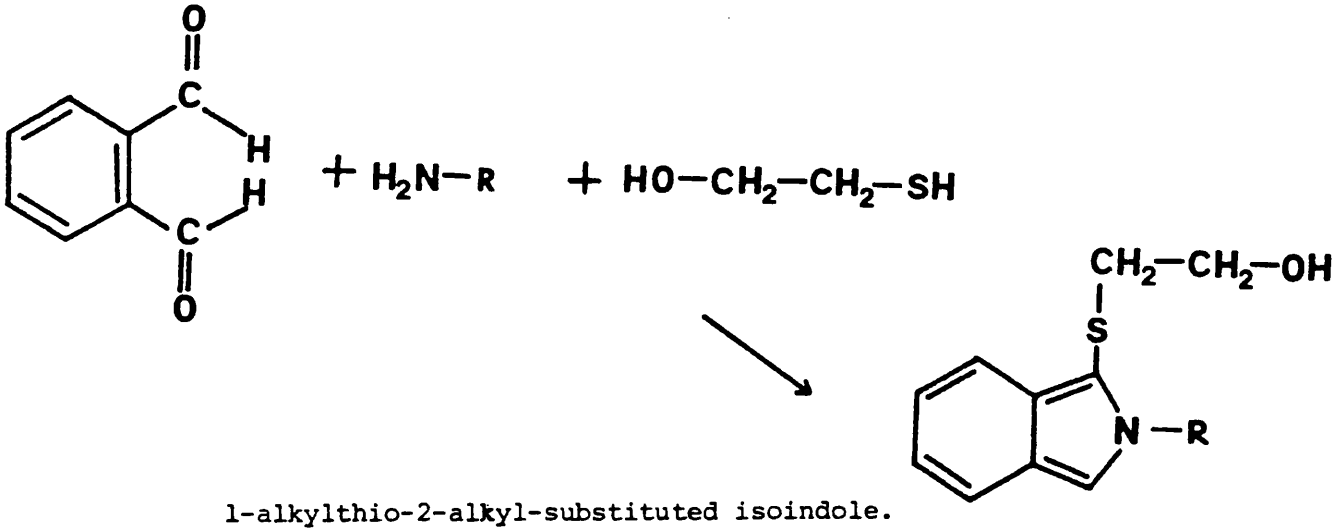
Recently, "high-performance" liquid chromatography has been suggested as a rapid, specific, and sensitive method for quantifying a variety of compounds, including antimicrobial agents (169). Its

principles are essentially the same as in liquid-chromatographic systems, in that separations are obtained by adsorption, partition, ion-exchange, or gel filtration. Pressures of 3.45 - 41.4 MPa (500-6000 lb/ $in^2$) may be applied to the column, and results obtained in minutes rather than hours or days as with conventional liquid chromatography.

The development of sensitive and specific detectors has been an important factor in the growth of usefulness of high-pressure liquid chromatography. Ultra-violet absorption is the most general method of detection in present use whilst fluorescence can provide even greater sensitivity and specificity. Since gentamicins contain neither a uv nor a fluorescent chromophore both these detection methods require the synthesis of suitable derivatives. In fact fluorescent derivatives have received the most attention.

Several reagents are currently available for the fluorescence detection of primary amines (171).

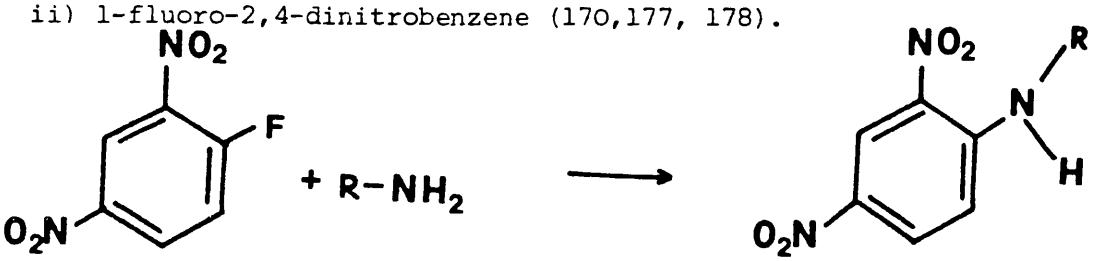
i) O-phthalaldehyde and 2-mercaptoethanol (172-175)



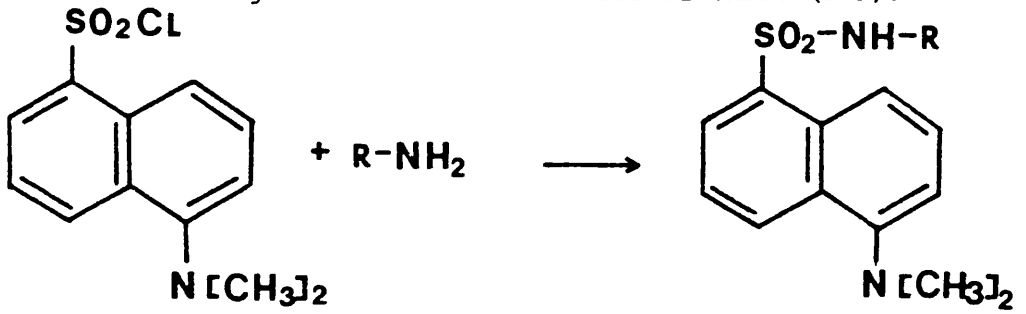

1-alkylthio-2-alkyl-substituted isoindole.

Freeman et al. (176) described a variation of this reaction in which the mercaptoethanol was replaced by thioglycollic acid.

ii) 1-fluoro-2,4-dinitrobenzene (170,177, 178).




iii) 5-dimethylaminonaphthalene - 1 - sulphonyl chloride (Dansyl chloride). This reagent can also be used for alcohols (179).




For HPLC the derivatives may either be prepared before injection onto the column (pre-column derivatisation) or the reaction may be carried out in a post column reaction coil after separation of the components (post-column derivatisation).

Advantages which have been claimed for pre-column derivatisation are summarised below (170):

(1) Unlike post-column derivatisation, no restrictions are imposed by the solvent system used as mobile phase. Both reaction conditions and chromatographic conditions can be chosen freely.

(2) The rates of the reactions are not usually limiting for in vitro reactions, whereas slow reactions can cause serious mixing and reaction-volume problems in post-column procedures.

(3) Derivatisation prior to chromatography can be used as a "pre-clean-up" step; selective reagents and extraction procedures can result in the elimination of much interference.

(4) Excess reagents can usually be eliminated easily in contrast to reactions after separation.

(5) Chromatographic properties such as retention time and resolution can be improved by a suitable choice of derivative.

(6) The choice of appropriate labelling reagents can result in suitable fragmentation patterns in mass spectrometric evaluation of the derivatives.

On the other hand, the limitations are:

(1) Side reactions might lead to more than one derivative and hence more than one peak for a single compound.

(2) The reactions must be quantitative, or at least reproducible, in both standard and analysis assays.

Alternatively the advantages of post-column procedures are (170):

(1) The formation of artifacts is not very likely.

(2) Different detection principles can be utilized simultaneously.

The limitations of this mode of operation are:

(1) The eluent can strongly influence the reaction medium.

(2) In HPLC, high flow-rates occur through small columns. Therefore the rates of post-column reactions must be relatively rapid (< 30 sec.), otherwise considerable dead-volume and reaction-volume problems ensue.

(3) A strict condition is that the derivatisation reagent must not be detectable under the conditions used for detection of the derivative.

Anhalt (107,180) has described a post-column derivatisation method in which the three major components were separated as ion

pairs with pentane sulphonic acid. A reversed phase column with chemically bonded octadecylsilane ($C_{18}$) was used, eluted with a mixture of sodium pentanesulphonate, sodium sulphate and aqueous acetic acid solution.

Post-column derivatisation with O-phthalaldehyde was carried out. Although these workers obtained reproducible results they found that the geometry of the post-column reaction coil did affect the fluorescence yield.

Several other workers have described methods which rely on pre-column derivatisation.

Peng et al. (179) and Chiou et al. (181) chromatographed the dansyl derivatives on a microparticulate reverse-phase column which was eluted with aqueous acetonitrile. This method separated gentamicin $C_1$ from $C_{1a}$ and $C_2$.

The UV absorbant 2,4-dinitrophenyl derivatives were separated on a reverse-phase column ($C_{18}$) by elution with tris (hydroxymethyl) aminomethane in water and acetonitrile but this method did not separate gentamicin $C_1$ and $C_2$ (177).

Maitra et al. (172) described the separation of all three major components as their O-phthalaldehyde derivatives on a reverse-phase column ($C_{18}$). The mobile phase was a mixture of methanol and water containing a specific amount of tripotassium ethylene diamine tetra acetate. Other aminoglycosides such as tobramycin and amikacin

did not interfere with the assay. A similar method has been described by Back et al. (182).

A modification of the O-phthalaldehyde reaction in which thioglycollic acid replaced mercaptoethanol has been proposed by Freeman et al. (176). By using these derivatives they separated all 3 major components and gentamicin $C_{2a}$ by ion-pair chromatography on a reverse-phase column. The mobile phase was a mixture of aqueous sodium heptane-sulphonate, anhydrous acetic acid and methanol. These HPLC systems have been used for the quantitative analysis of the major components by the use of a suitable standard gentamicin preparation.

No derivatisation is required if a refractive index detector is used (107). This is relatively insensitive but is most suitable for preparative work.

1.12.6 Gas-liquid chromatography of gentamicin

The gas-liquid chromatographic separation of gentamicin requires the derivatisation of the -OH and $-NH_2$ groups to yield sufficiently volatile and heat stable compounds.

Mayhew and Gorbach (183, 184)reported a rapid analysis of gentamicin in serum in which -O- silylated and -N- acetylated derivatives of gentamicin were examined on OV 17 at 262°C. Gentamicin $C_{1a}$ was resolved but $C_2$ and $C_1$ gave a single peak. Normal serum constituents did not interfere with the chromatograms and neither did other drugs and antibiotics administered concurrently

with gentamicin therapy. Quantitation was based upon the relative response of gentamicin to a fixed amount of the internal standard, kanamycin A. Isothermal chromatographic analysis time per single serum specimen was approximately 50 minutes.

## Introduction to the Research Problem

The many chromatographic, structural and biological studies just described have yielded a large body of knowledge concerning this antibiotic. However, many important questions remain. In the analytical field these include:

### 1) Pharmacopoeial standards

The monograph for gentamicin sulphate (British Pharmaceopaeia, 1980) (185) specifies proton nuclear magnetic resonance (NMR) spectrometry as a method of monitoring proportions of the main components. The purity is controlled by thin-layer chromatography Sulphate determination is carried out by a gravimetric method with barium chloride solution and water content by Karl Fischer titration. The total potency of the compound is determined by biological assay.

The United States Pharmacopoeia, 1980 (186) specifies paper chromatography followed by bioautography as a method for controlling the proportions of gentamicin major components. Water content is determined by loss on drying.

### 2) The manufacture and quality control of formulations

Total potency and purity are controlled according to the monograph. In some cases an extraction method has to be used in order to remove other interfering substances.

3) Accurate determination of serum levels

The aim of antibiotic therapy is to obtain a high enough concentration of the drug at the infected site to kill or inhibit the growth of the microbe. It would therefore be logical to measure the concentration of the drug in the affected tissue to ensure that the dose administered is adequate. However, such measurements are technically difficult to perform (it may not be feasible to obtain samples, it is difficult to recover drug from the tissue in a standardised manner) and concentration determinations are therefore usually done in samples obtained from serum and other readily available body fluids.

Not only must the clinician be sure that the administered antibiotic reaches effective concentrations in the body; he must also guard against undesirable side effects. Gentamicin possesses nephrotoxicity, ototoxicity and may cause neuromuscular blockade. Usually the appearance of toxic damage seems to be related to the concentration of the drug in the blood stream.

Thus, from the aspects of safety and efficacy, it is necessary to monitor gentamicin concentration during therapy. This is especially true when an abnormal rate of elimination can be suspected, for example in patients with renal diseases and in children.

Ideally, an assay technique for gentamicin concentration should be rapid, so that the answer is available prior to next dosage; it should be accurate; and it should be specific so that other antibiotics concomitantly administered do not cause

a falsely high value.

Several methods have been developed to determine serum level of gentamicin. They include microbiological assay, enzymic assay, immunoassay and high-performance liquid chromatography.

(4) The chemical nature and biological activity of the minor components

Many workers have observed minor components in gentamicin samples (158, 159). Many of these are unidentified and their biological activity (if any) is unknown. Thus the main objectives of the project were:

(a) To isolate as many as possible of the components of commercial gentamicin.

(b) To identify some of the minor components and examine their biological activity.

(c) To use the pure major components as standards in developing an assay for gentamicin suitable for use with commercial raw materials, formulations and serum.

The recent work on the separation of the gentamicin complex by HPLC shows its potential as an analytical tool. Since it is possible to separate the major components of the gentamicin complex it can potentially be used to determine component ratios in addition to the total amount of gentamicin. In addition to high specificity and sensitivity a major advantage is the speed of assay. Thus one of the objectives of this project has been to apply HPLC techniques to the assay of gentamicin in commercial raw materials, formulations and serum and to compare this method with existing official assays.

# CHAPTER 2

## Chromatographic separation and characterisation of the components of commercial gentamicin C

### Introduction

As mentioned in the previous chapter the gentamicin C complex has been shown to consist of at least three major components. The present chapter describes the chromatographic separation of gentamicin by paper chromatography, thin-layer chromatography, column chromatography, and preparative high-pressure liquid chromatography and subsequent spectroscopic investigations.

### Materials and Methods

#### 2.1 Separation of the gentamicin C complex

##### Materials

Gentamicin sulphate (mixture) was donated by Nicholas Laboratories Limited. Solutions of 20 mg/ml and 50 mg/ml in water were used for TLC and paper chromatography respectively. Chloroform, methanol, ammonia solution, ethanol, acetone and glacial acetic acid were obtained from Fisons Scientific Apparatus Limited. Ninhydrin, silica gel for chromatographic adsorption, potassium permanganate, sodium hydroxide and pyridine were obtained from BDH Chemicals Limited on silica gel H and silica gel 60 were obtained from Anderman and Co. Limited (U.K. distributors of E. Merck Laboratory Chemicals). Amberlite IRA-400 was obtained from Rohm and Hass Co.

Methods

2.1.1 Paper chromatography (185)

Paper chromatography was carried out by a descending technique using Whatman No. 20 paper.

The lower layer of a mixture of chloroform-methanol-concentrated ammonium hydroxide (sp. gr. 0.88)-water (10:5:3:2) was placed in the solvent trough. The chamber was saturated with solvent vapour by placing portions of both upper and lower phases in the bottom of the tank for 24 hours before use. 2 µl of gentamicin sulphate solution was applied to the paper and developed for 5 hours.

After drying at 105°C for one hour, the paper was sprayed with a freshly prepared solution of ninhydrin (0.25 g) in a mixture of equal volumes of pyridine and acetone and then heated for 2 minutes at 105°C. The gentamicin components were visible as purple spots on a white background.

The $R_F$ value was calculated from

$$\frac{\text{distance moved by the solute}}{\text{distance moved by the solvent}}$$

2.1.2 Analytical thin-layer chromatography (164).

Adsorbant

Silica gel 60 plates (20 x 20 cm) 0.25 mm thickness were heated for 1 hour at 135°C prior to use.

Solvent

The lower layer of a mixture of methanol-chloroform-concentrated

ammonium hydroxide (sp. gr. 0.88) (1:1:1). Care was taken to ensure that the chamber was saturated with solvent vapour. The plate was subjected to a single development to a height of 15 cm.

Visualisation

After drying at 135°C, the plate was sprayed with a freshly prepared solution of ninhydrin (1.0 g) in a mixture of 95% ethanol (50 ml) and glacial acetic acid (10 ml) and then heated for 15 minutes at 135°C. The gentamicin components were visible as purple spots on a white background.

This TLC system was affected by variation in temperature. To obtain reproducible results the chamber was placed in a thermostatically controlled water bath at 25 ± 0.2°C. The solvent was changed after 3 days use due to the loss of ammonia altering the solvent composition. This TLC system was used to monitor the results of subsequent preparative procedures.

2.1.3 Preparative thin-layer chromatography

The method used was the same as for analytical TLC, except that silica gel H was used to prepare layers of 1.0 mm thickness. After development, the plates were dried at room temperature and a small portion of the plate sprayed with 1% potassium permanganate solution whilst the rest of the plate was masked. The gentamicin components gave yellow bands on a pink background. The unsprayed portions of the plate corresponding to these bands were scraped off and extracted with water for 3 hours. The solutions were concentrated and monitored by analytical TLC.

In order to reduce losses due to decomposition, a similar plate was run in which the silica gel H was slurried with 0.05 M phosphate buffer, pH 7.0 instead of water.

2.1.4 Column chromatography

2.1.4.1 Formation of gentamicin base

Amberlite IRA 400 (Cl form) (30 g) was converted to the $OH^-$ form by passing 1N sodium hydroxide through the column until the eluate was basic. It was then washed with water until the eluate was neutral.

Gentamicin sulphate (300 mg) dissolved in water (0.5 ml) was applied onto the column and eluted with distilled water. The pH of the eluate was monitored and all the alkaline fractions were collected and combined. This was lyophilized to obtain gentamicin base as a white solid.

2.1.4.2 Column chromatography using silica gel (60 mesh) (102)

Silica gel (25 g) was slurried with the lower phase of th solvent system chloroform-isopropnaol-17% w/v ammonia solution (2:1:1) and packed into a chromatographic glass column (1 cm i.d.). Gentamicin base mixture (200 mg) was dissolved in the same solvent (0.3 ml) and chromatographed using a flow rate of about 0.5 ml/mm. Fractions (1 ml) were collected automatically and monitored by TLC.

2.1.4.3 Column chromatography using silica gel H (TLC grade)

Silica gel H (16 g) was slurried with the lower phase of a mixture of chloroform-isopropnaol-17% w/v ammonia solution (2:1:1) and packed into a chromatographic glass column (2.5 cm. i.d.).

Gentamicin base mixture (200 mg) was dissolved in the same solvent (0.3 ml), applied onto the column and eluted with this solvent. The column was run at the rate of 0.3 ml/min and 3 ml fractions were collected, automatically. After 800 ml the elution was continued with a mixture of chloroform-isopropanol-concentrated ammonia solution (2:1:1). 6 ml fractions were collected. After 400 ml of this solvent another change was made to the lower phase of a mixture of chloroform-isopropanol-concentrated ammonia solution (1:1:1). The fractions were monitored by TLC and those containing pure components were combined, the organic solvent removed under reduced pressure and the water removed by lyophilization.

2.1.4.4 Preparative high-pressure liquid chromatography

| | |
|---|---|
| Instrument | Jobin Yvon Chromatospac Prep. |
| | Division d'instruments S.A. |
| Column | 4 cm i.d. |
| Support | Lichroprep$^{R}$ Si 60 |
| | particle size 15 - 25 µm |
| | weight 200 g |
| Flow rate | 20 ml/min |
| Pressure | 7.2 bar |

Gentamicin base mixture (3.5 g) in the mobile phase (4 ml) was applied onto a column and eluted with the same mobile phase as for column chromatography (2.1.4.3). The instrument was equipped with a refractive index detector but as this was not sensitive enough to detect minor components, 10 ml fractions were collected automatically and monitored by TLC.

## 2.2 Spectroscopic examination of gentamicin components

### 2.2.1 Ultraviolet and visible spectra

Ultraviolet and visible spectra were recorded using Perkin-Elmer 550S UV-VIS double beam, recording spectrometer.

### 2.2.2 Infrared spectra

Infrared spectra were determined on a Unicam SP 200G Grating Infrared Spectrophotometer. The sample was prepared as a Nujol mull.

### 2.2.3 Mass Spectrometer

Field desorption mass spectra were determined by Dr. D.E. Games at the Department of Chemistry, University College, Cardiff, using a Varian CH-5D mass spectrometer on-line to a Varian 620 i computer. Methyl alcohol was employed as solvent, the source temperature was 80°C and optimum emitter currents were 17 to 18 mA.

Chemical ionization mass spectra were determined by Dr. R. Black at the Chemical Defence Establishments, Porton Down, U.K., using a V.G. Micromass 7070 mass spectrometer. Isobutane was used as the reagent gas at a source pressure of 0.5 Torr. The source temperature was 200°C.

Electron impact mass spectra were determined by Mr. C. Cryer at the University of Bath, Department of Chemistry using an AEI single focussing mass spectrometer model No. MS12. The ionizing potential was 12 eV and the ion source temperature 180°C.

2.2.4 Nuclear Magnetic Resonance

100 MHz proton NMR spectra were determined by Mr. D. Wood on a JEOL P.S. 100 NMR spectrometer (continuous wave), 90 MHz proton and carbon-13 NMR spectra were determined by Mr. J. Kountourellis on a JEOL FX 90Q Fourier Transform NMR spectrometer, at the University of Bath, School of Pharmacy and Pharmacology.

Results and Discussion

a) Separation of gentamicin C complex

1) Physical descriptions

As free bases, gentamicin C components are white, odourless, amorphous hygroscopic solid. They are highly soluble in water and methanol, less so in ethanol, and insoluble in acetone, ether and other less polar solvents.

2) Paper chromatography

Table 5 shows the separation of gentamicin C by this method.

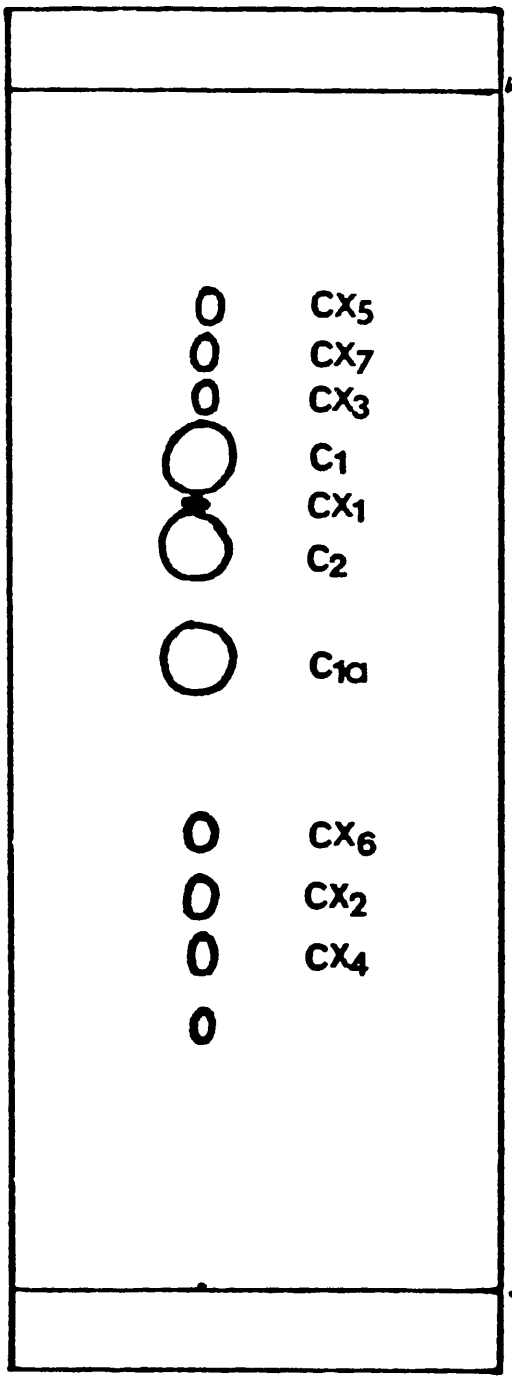
Table 5. Paper chromatography of gentamicin C

| Compound | $R_F$ value |
|---|---|
| Gentamicin $C_1$ | 0.56 |
| Gentamicin $C_2$ | 0.48 |
| Gentamicin $C_{1a}$ | 0.34 |

The three major components were well separated but no minor components were observed. This method was time consuming (5 hours) and was not suitable for routine use to monitor the results of column chromatography.

3) Thin-layer chromatography

Figure 14 represents a chromatogram of the gentamicin C complex and the $R_F$ value of each individual components in comparison with the method of Wilson et al.(158).




| Compounds | $R_F$ values | $R_F$ values (Wilson et al.) |
|---|---|---|
| $CX_5$ | 0.85 | |
| $CX_7$ | 0.80 | |
| $CX_3$ | 0.75 | |
| $C_1$ | 0.69 | 0.44 |
| $CX_1$ | 0.65 | |
| $C_2$ | 0.61 | 0.39 |
| $C_{1a}$ | 0.52 | 0.35 |
| $CX_6$ | 0.37 | |
| $CX_2$ | 0.31 | |
| $CX_4$ | 0.18 | |

It can be seen that the three major components were well separated as were a number of minor components. Reproducibility of the method was obtained when the chromatographic chamber was placed in a thermostatically controlled water bath at 25 $\pm$ 0.2°C.

The method of Wilson et al.(158) differs from the one presented here in that he used commercially coated plates (E.M. Laboratories Inc., New York) and ambient temperatures. The solvent system is the same. He observed six minor components, one less polar than gentamicin $C_1$ and five more polar than gentamicin $C_{1a}$. These cannot be correlated with any of the minor components observed during the present study owing to the wide differences in $R_F$ values.

4) Preparative thin-layer chromatography

Both buffered and unbuffered plates gave very low recovery of gentamicin components. These were not pure and the evidence suggested that decomposition had occurred during the preparative procedure.

5) Column chromatography

The results of the two column and the high-pressure liquid chromatographic separations of gentamicin are shown in Tables 6, 7 and 8.

Table 6. The separation of gentamicin C using column grade silica gel

| Solvent | | Compound |
|---|---|---|
| Chloroform:isopropanol:17% ammonia (2:1:1) | | |
| Fractions | 55 - 59 ml | $C_1$ |
| | 58 - 70 | $C_2$ |
| | 60 - 70 | $C_{1a}$ |

Table 7. The separation of gentamicin C using TLC grade silica gel.

| Solvent | | Compound |
|---|---|---|
| Chloroform:isopropanol:17% ammonia (2:1:1) | | |
| Fractions | 111 - 123 ml | $CX_5$ |
| | 135 - 147 | $CX_7$ |
| | 156 - 204 | $CX_3$ |
| | 225 - 318 | $C_1$ |
| | 309 - 321 | $CX_1$ |
| | 345 - 450 | $C_2$ |
| | 510 - 675 | $C_{1a}$ |
| Chloroform:isopropanol: conc. ammonia (2:1:1) | | |
| | 120 - 150 | $CX_6$ |
| | 180 - 240 | $CX_2$ |
| Chloroform:isopropanol: conc. ammonia (1:1:1) | | |
| | 360 - 600 | $CX_4$ |

Table 8. The separation of gentamicin C by preparative high-pressure liquid chromatography

| Solvent | | Compound |
|---|---|---|
| Chloroform:isopropnaol:17% ammonia (2:1:1) | | |
| Fractions | 2,200 - 2,600 ml | $CX_5$ |
| | 2,500 - 2,700 | $CX_7$ |
| | 2,800 - 3,600 | $CX_3$ |
| | 3,500 - 5,200 | $C_1$ |
| Chloroform:isopropanol:conc. ammonia (2:1:1) | | |
| Fractions | 600 - 1,250 ml | $C_2$ |
| | 1,200 - 2,700 | $C_{1a}$ |
| | 3,700 - 4,000 | $CX_6$ |
| | 5,180 - 6,180 | $CX_2$ |
| Chloroform:isopropanol: conc. ammonia (1:1:1) | | |
| Fractions | 500 - 2,000 ml | $CX_4$ |

It can be seen that the relatively coarse column grade silica gel only partially separated the mixture and gave rise to mainly mixed fractions.

A similar column containing TLC grade silica gel gave much better resolution and allowed pure samples of all the major components and minor components which were designated $CX_1$-$CX_7$ to be isolated. This column proved to be reproducible from run to run. However the column had a maximum capacity of 200 mg of gentamicin and took about 1 week to run. Although the HPLC system had a slightly less good resolution it enabled 3.5 g of gentamicin mixture to be separated in less than one day.

Both columns gave the elution order which would be predicted from the similar TLC system.

b) Spectroscopic examination of gentamicin components

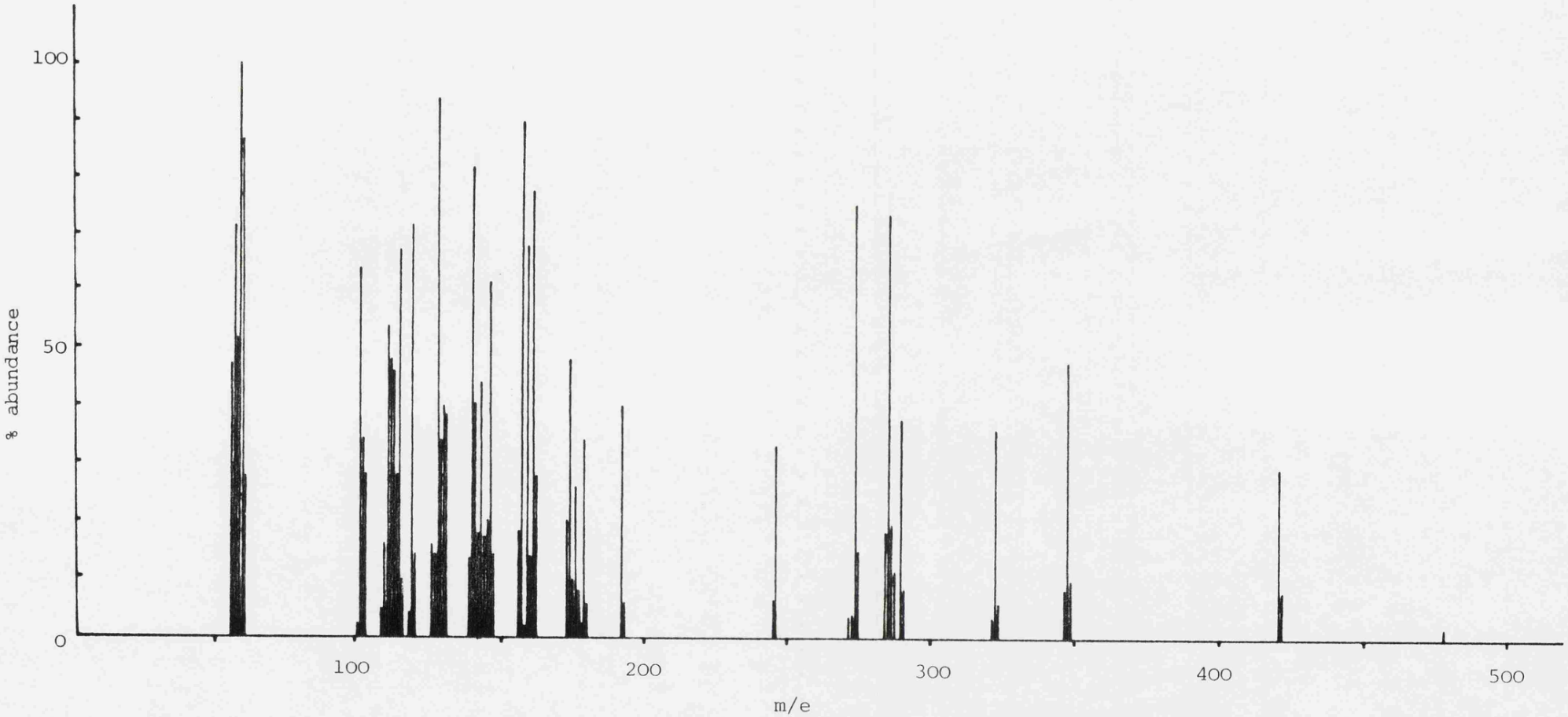
None of the gentamicin components exhibits absorption in the visible or ultraviolet regions of the spectrum. Infrared spectra were identical for all. The only notable feature was a broad peak in the 3,000 - 4000 $cm^{-1}$ region due to O-H and N-H stretching.

1) Gentamicin $C_1$, $C_2$ and $C_{1a}$

Field desorption mass spectra of these components show a quasimolecular ion $(M + H)^+$ as the only intense line in each spectrum.

By contrast electron impact spectra give at best a very weak $M^+$ and many fragment ions. Figure 15 shows the electron impact

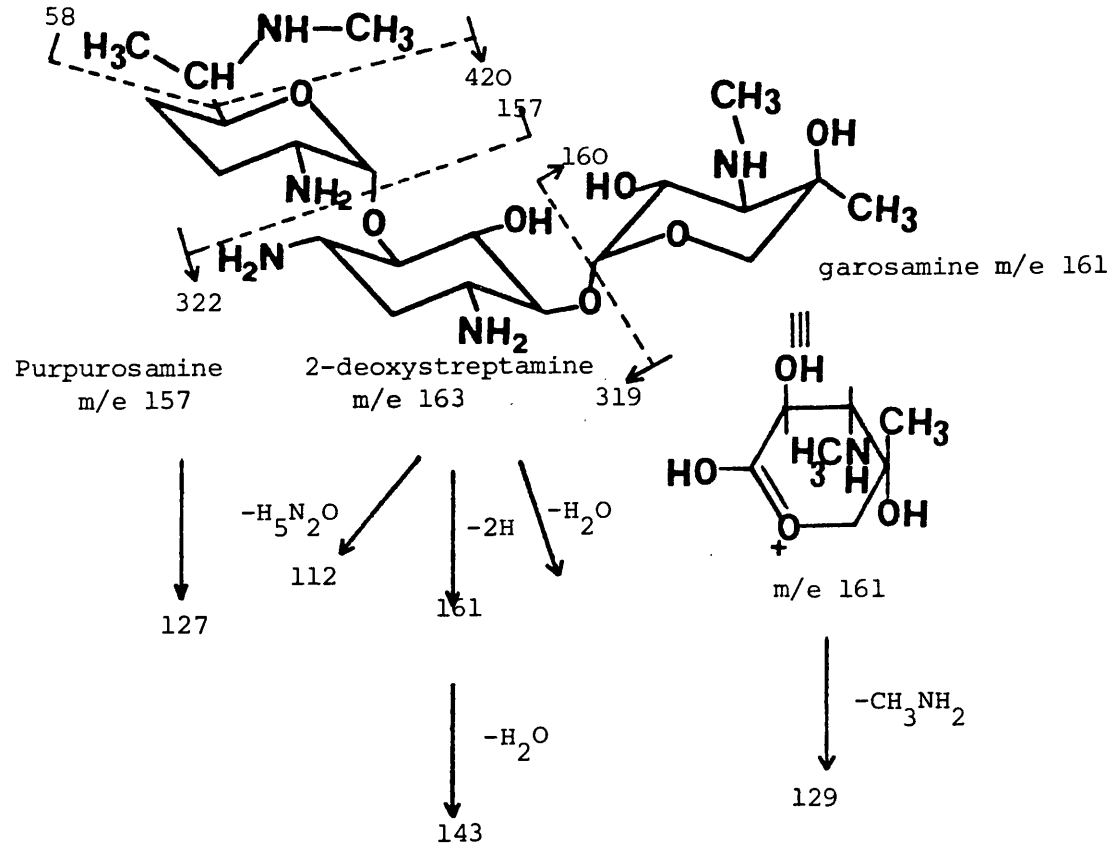
Figure 15. Electron impact mass spectrum of gentamicin $C_1$



spectrum of gentamicin $C_1$.

Daniels et al.(97) suggested the fragmentations shown in the scheme in Figure 16. The fragmentation patterns of gentamicin $C_2$ and $C_{1a}$ were similar to that of $C_1$ except for the difference in mass of the purpurosamine ring, and the lack of the fairly prominent peak at m/e 420 due to cleavage of the purpurosamine side chain. In particular they pointed out that fragments with m/e 191, 173, 163 and 145 were characteristic of 2-deoxystreptamine containing antibiotics.

Figure 16. Daniel's (97) fragmentation scheme for gentamicin $C_1$



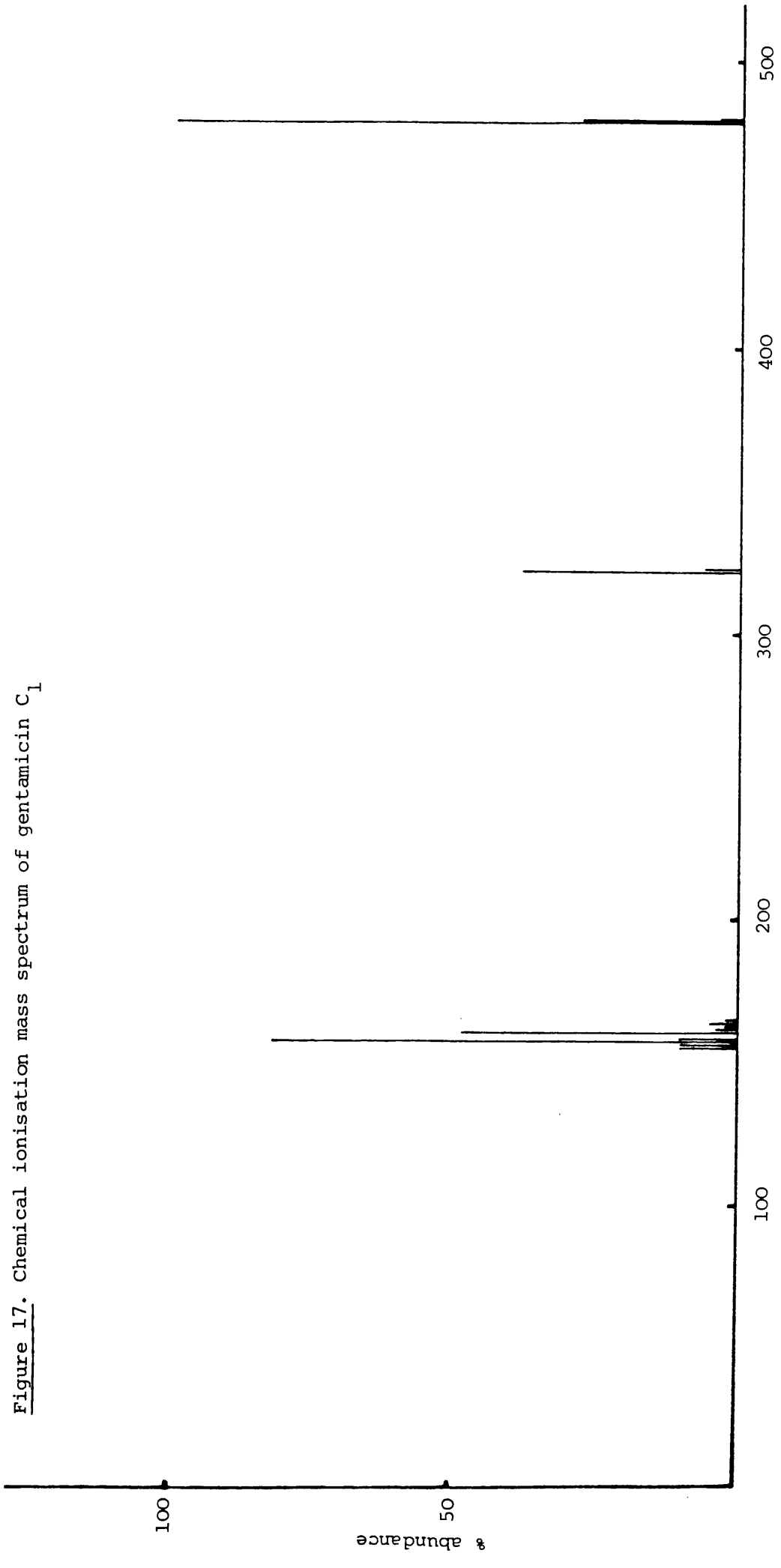
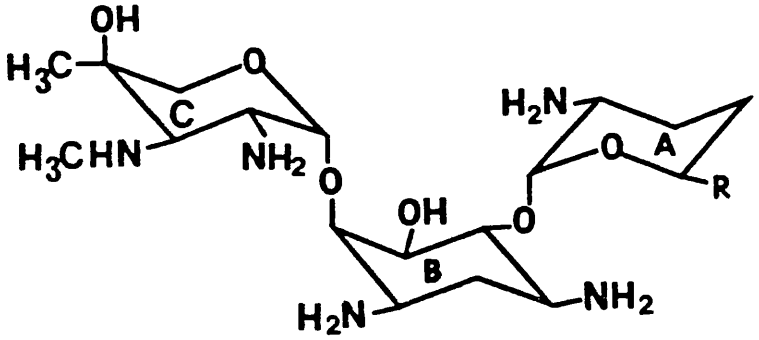



Figure 17. Chemical ionisation mass spectrum of gentamicin $C_1$

Fragments with m/e 161 and 163 are characteristic of the garosamine and 2-deoxystreptamine rings respectively. For the purpurosamine ring, fragments with m/e 157, 143 and 129 are characteristic of gentamicin $C_1$, $C_2$ and $C_{1a}$ respectively. The garosamine moiety may also give rise to a peak of m/e 129. Figure 17 shows the chemical ionisation mass spectrum of gentamicin $C_1$.

The chemical ionisation mass spectra show a prominent $[M + H]^+$ peak and a somewhat simpler fragmentation pattern which chiefly reflects cleavage at the glycosidic bonds as summarised in Figure 18.

Figure 18.




R = -CH(CH$_3$) - NH - CH$_3$ gentamicin $C_1$

R = -CH(CH$_3$) - NH$_2$ gentamicin $C_2$

R = -CH$_2$ - NH$_2$ gentamicin $C_{1a}$

Figure 18 continued

| | m/e (% abundance) | | |
|---|---|---|---|
| | GENTAMICIN $C_1$ | GENTAMICIN $C_2$ (155) | GENTAMICIN $C_{1a}$ (155) |
| $[M + H]^+$ | 478 (100) | 464 (100) | 450 (100) |
| B + C | 322 (38) | 322 (68) | 322 (67) |
| C | 160 (48) | 160 (7) | 160 (22) |
| B | 163 (5) | 163 (5) | 163 (30) |
| A | 157 (82) | 143 (17) | 129 (22) |

Cleavage of garosamine from the gentamicins gives rise to a weak ion at m/e 160 in all of the spectra whilst cleavage of the purpurosamine moiety gives the characteristic ions of m/e 157, 143 and 129.

It is noteworthy that there are no prominent peaks at m/e 319, 305, 291 corresponding to a protonated gentamicin moiety (rings A + B) whereas the protonated garosamine moiety (rings B + C) gives a very prominent peak at m/e 322.

It is clear from the mass spectra that these major components of gentamicin differ in the degree of methylation of the purpurosamine rings. This is confirmed by the proton NMR spectra shown in Figures 19, 20 and 21. These spectra are in agreement with those obtained by Cooper *et al.* (98) and Daniels *et al.* (106) who assigned the peaks as follows. All the components show a singlet at δ1.18 which conforms to a $C\text{-}CH_3$ group (4"). The $N\text{-}CH_3$ group (3") is shown by a singlet at δ2.50. The anomeric protons occur as doublets at

Figure 19. PMR spectrum of gentamicin $C_1$

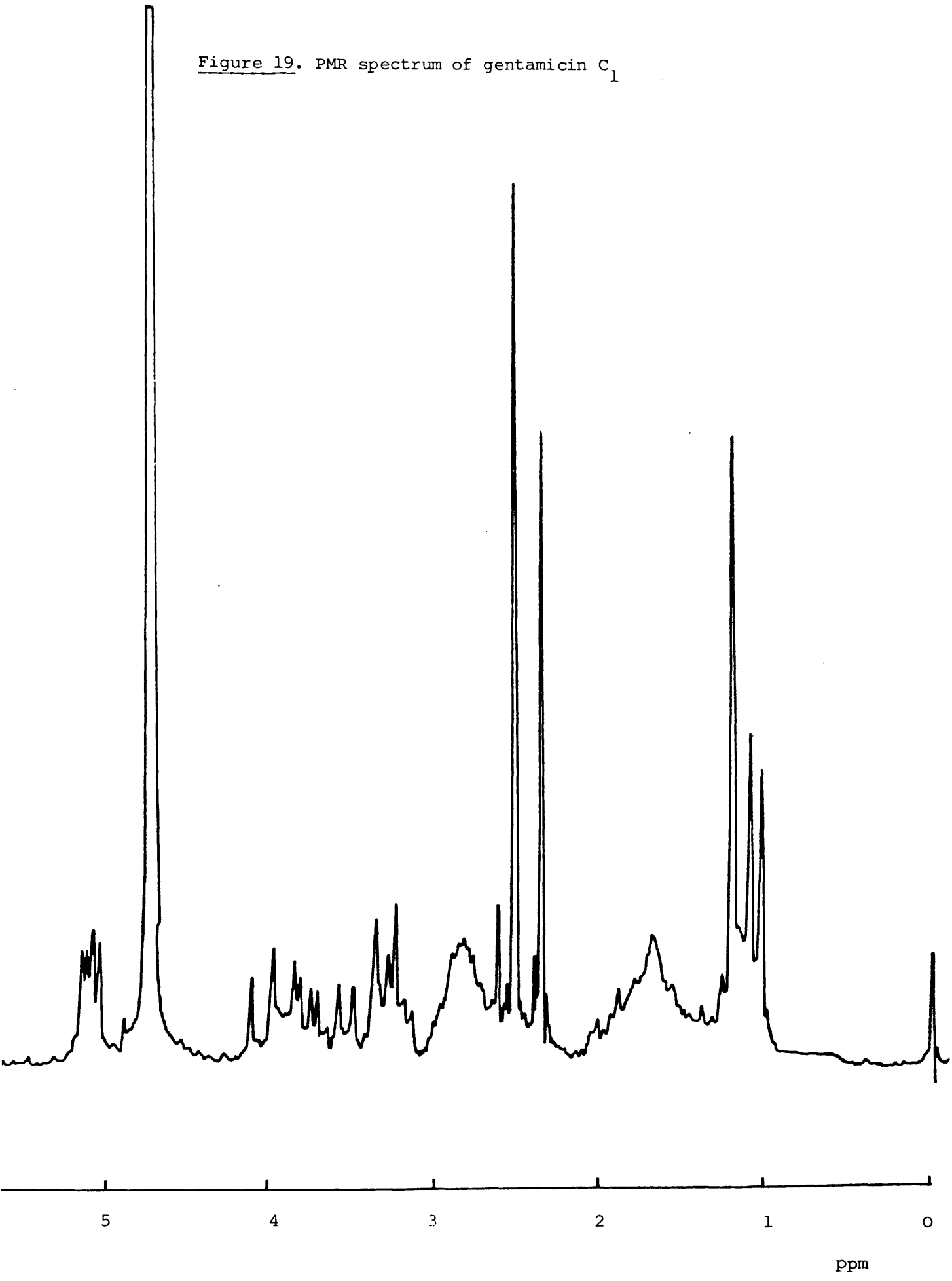


Figure 20. PMR spectrum of gentamicin $C_2$

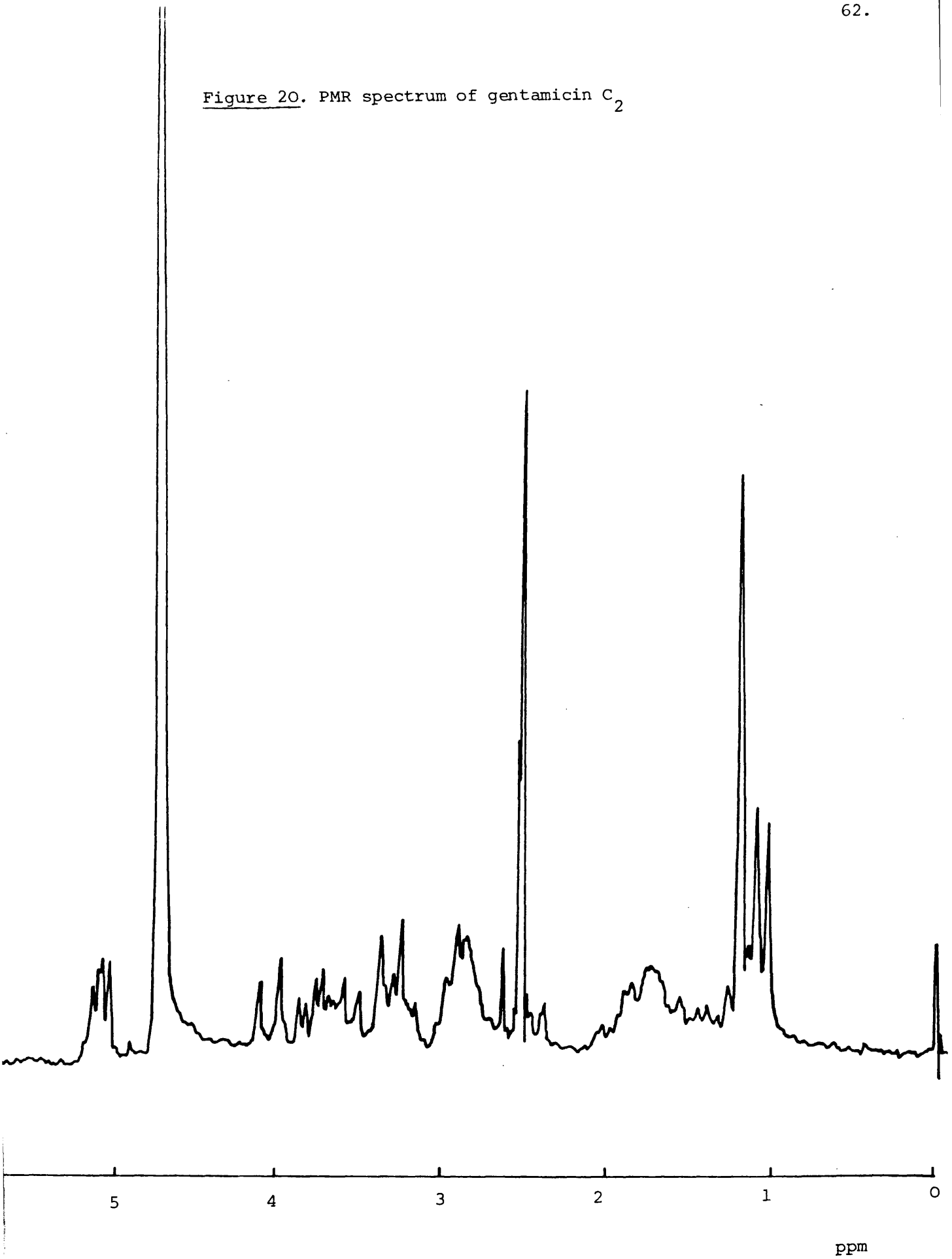
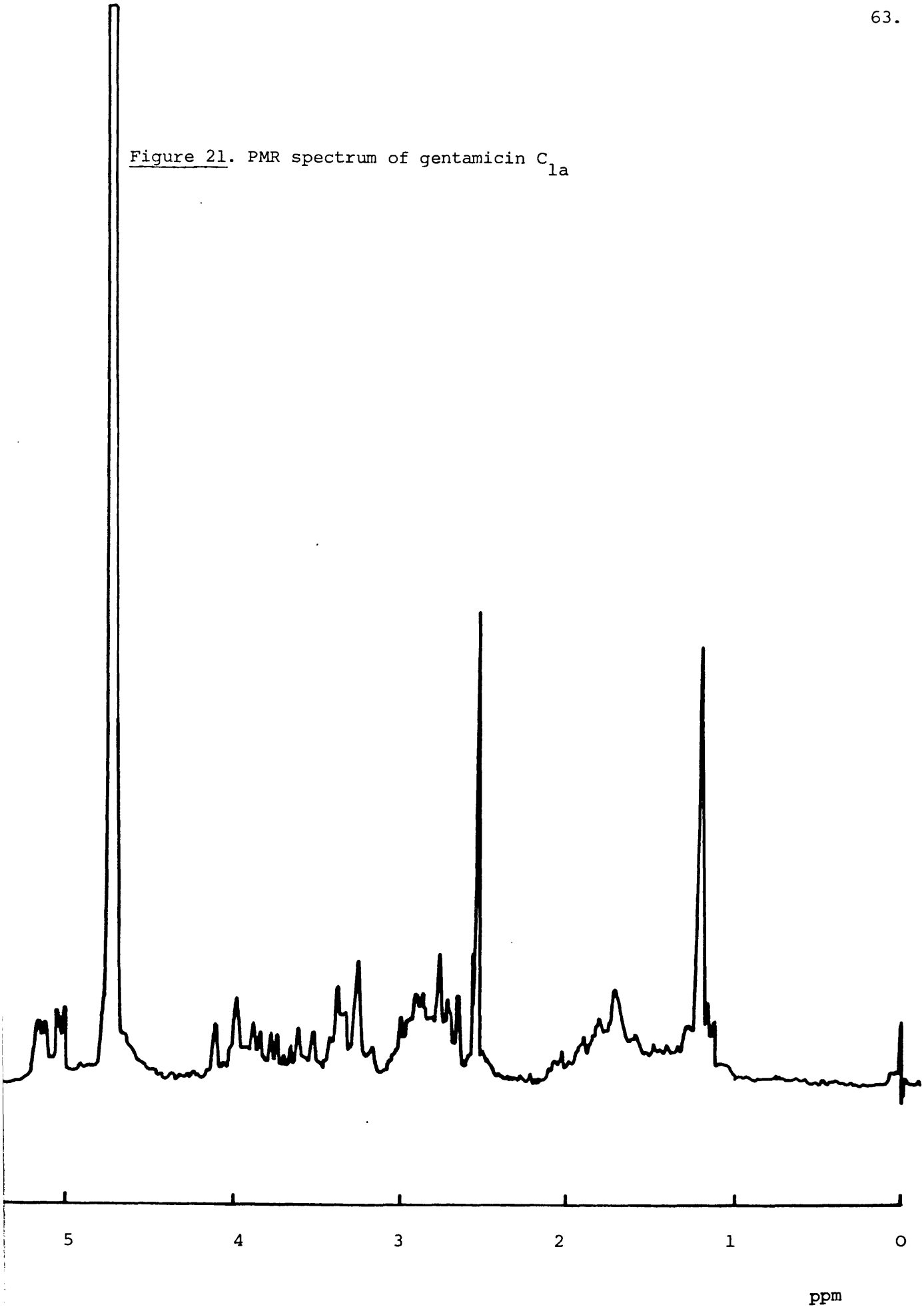


Figure 21. PMR spectrum of gentamicin $C_{1a}$



δ5.04 and δ5.12 (1" and 1' proton respectively). Doublets of 5"-equatorial and axial protons appear at δ4.02 and δ3.28 respectively. A quartet at δ3.76 and a doublet at δ2.54 indicate the presence of the 2"-equatorial and 3"-proton respectively. The 2-equatorial proton in 2-deoxystreptamine ring is characterised by a double of triplets at δ1.94.

The differences between gentamicin $C_1$, $C_2$ and $C_{1a}$ lie in their degree of methylation at 6'-position of purpurosamine ring. Gentamicin $C_{1a}$ has the basic unmethylated ring whilst gentamicin $C_2$ shows a doublet at δ1.04 corresponding to the secondary methyl group at C-6'. Gentamicin $C_1$ also has this peak plus a three proton singlet at δ2.34 due to the $-NH-CH_3$ group.

Figure 22 compares the PMR spectra of gentamicin base mixture and its sulphate salt. The major differences are the resonances of the anomeric protons, H-2" proton, H-5" equatorial and axial protons as shown in Table 9. One half of the doublet assigned to the secondary C-methyl group is masked by the tertiary C-methyl signal (δ1.36) in the spectrum of the sulphate.

Table 9. Resonances of some of the protons in the free base and sulphate salt.

| Proton | Resonance peak (δ,ppm) | |
|---|---|---|
| | free base | sulphate |
| H - 1' | 5.12 | 5.92 |
| H - 1" | 5.04 | 5.14 |
| H - 5" eq | 4.02 | 4.04 |
| H - 5" ax | 3.28 | 3.50 |
| H - 2" | 3.76 | 4.24 |
| $-\overset{|}{C}-CH_3$ | 1.18 | 1.36 |
| $-NH-CH_3$ | 2.48 | 2.96 |

Figure 22. PMR spectrum of gentamicin base mixture

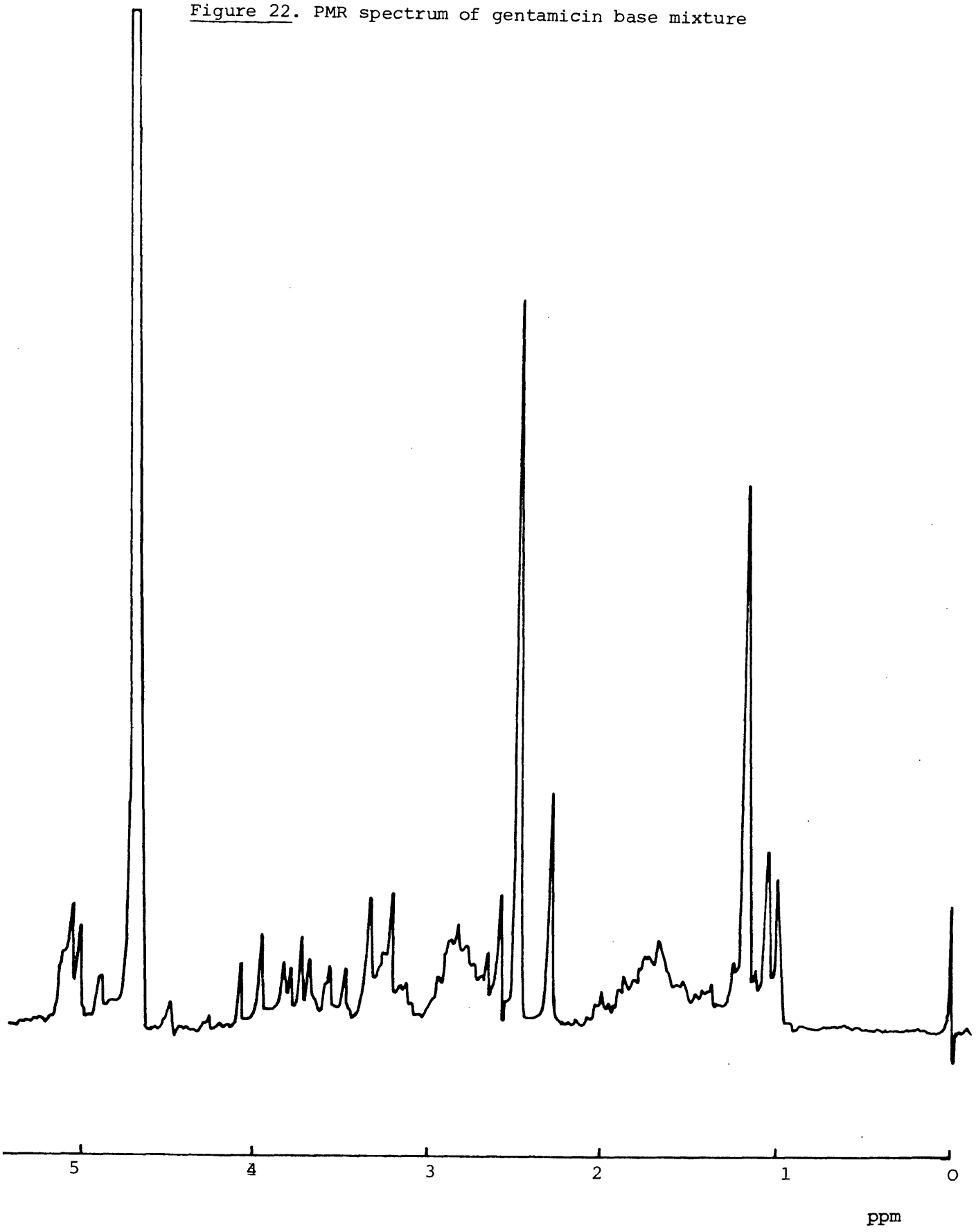
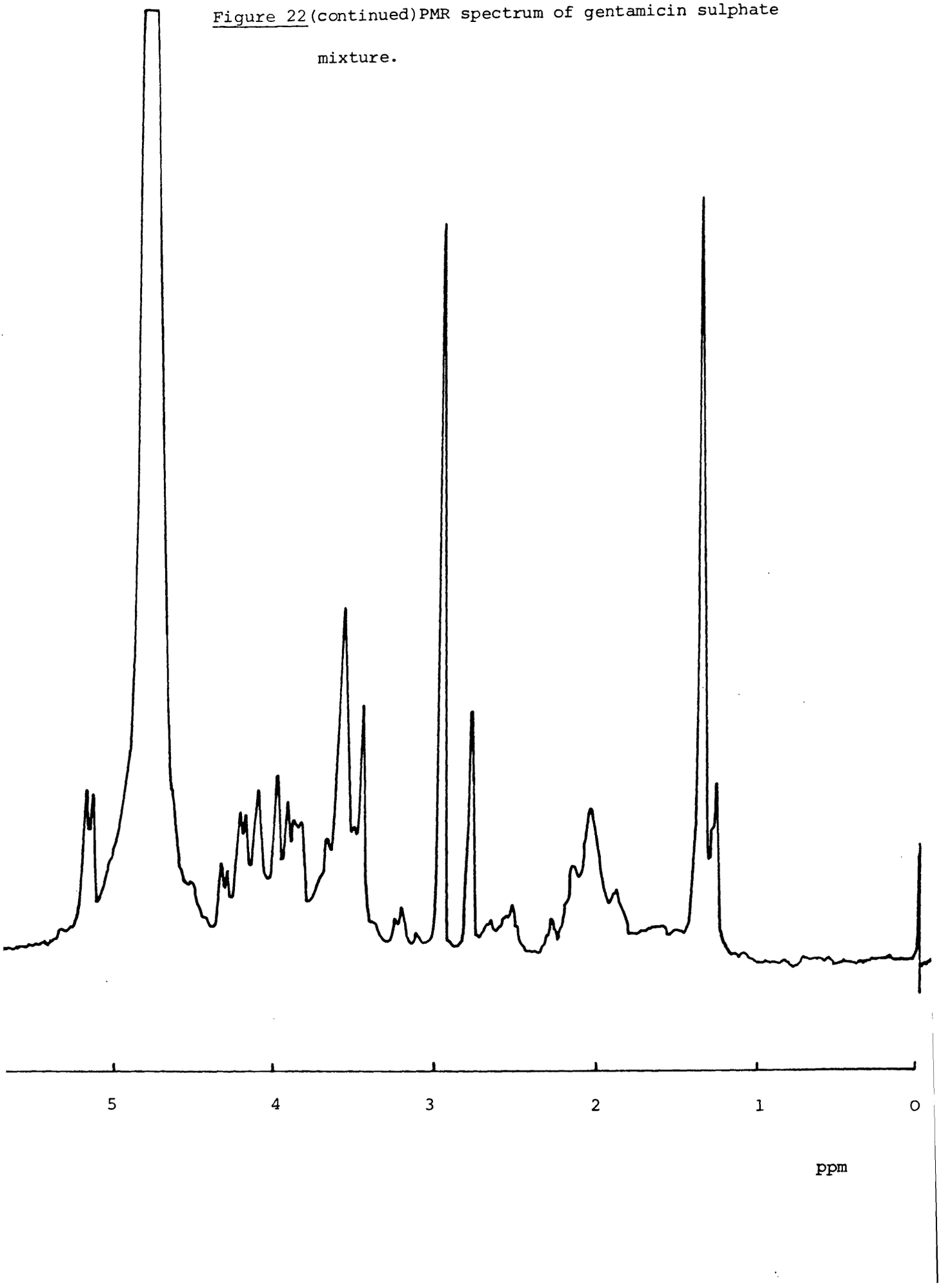


Figure 22 (continued) PMR spectrum of gentamicin sulphate mixture.



Figures 23, 24 and 25 show $^{13}C$ NMR spectra of gentamicin $C_1$, $C_2$ and $C_{1a}$ respectively and Table 10 shows the allocation of chemical shifts according to a study by Morton *et al.* (103)

The differences in chemical shifts between gentamicin free base mixture and its sulphate salt are shown in the Figure 26 and Table 11.

Figure 23. $^{13}C$ NMR spectrum of gentamicin $C_1$

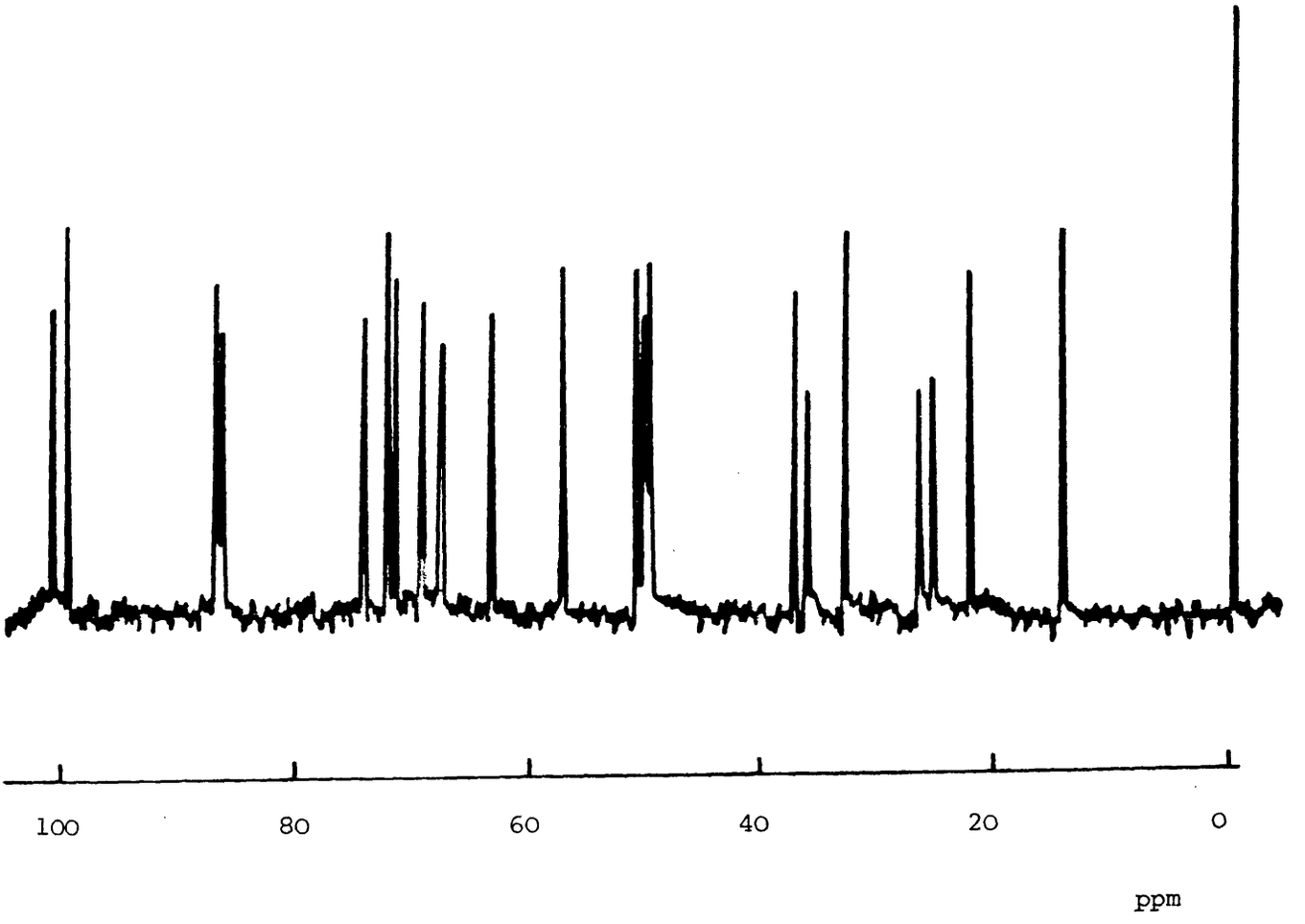


Figure 24. $^{13}C$ NMR spectrum of gentamicin $C_2$

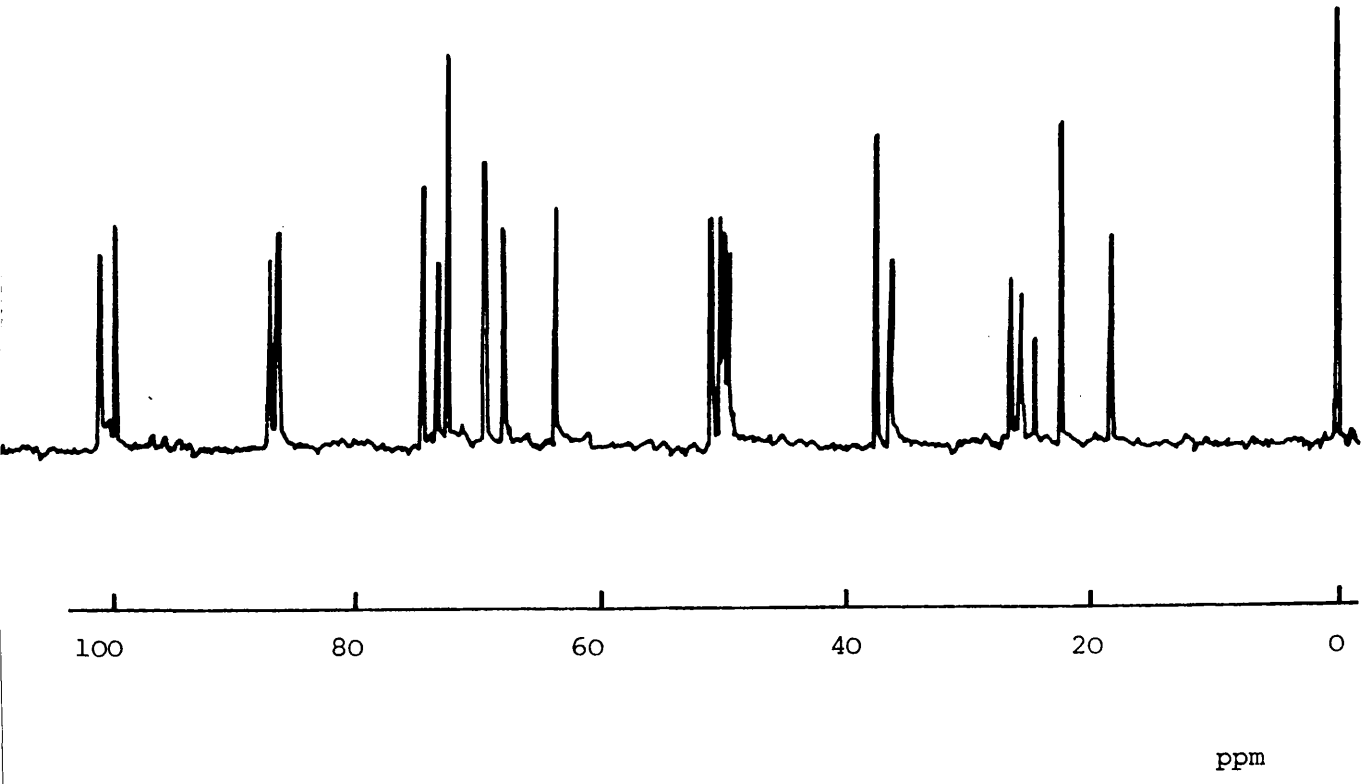


Figure 25. $^{13}C$ NMR spectrum of gentamicin $C_{1a}$

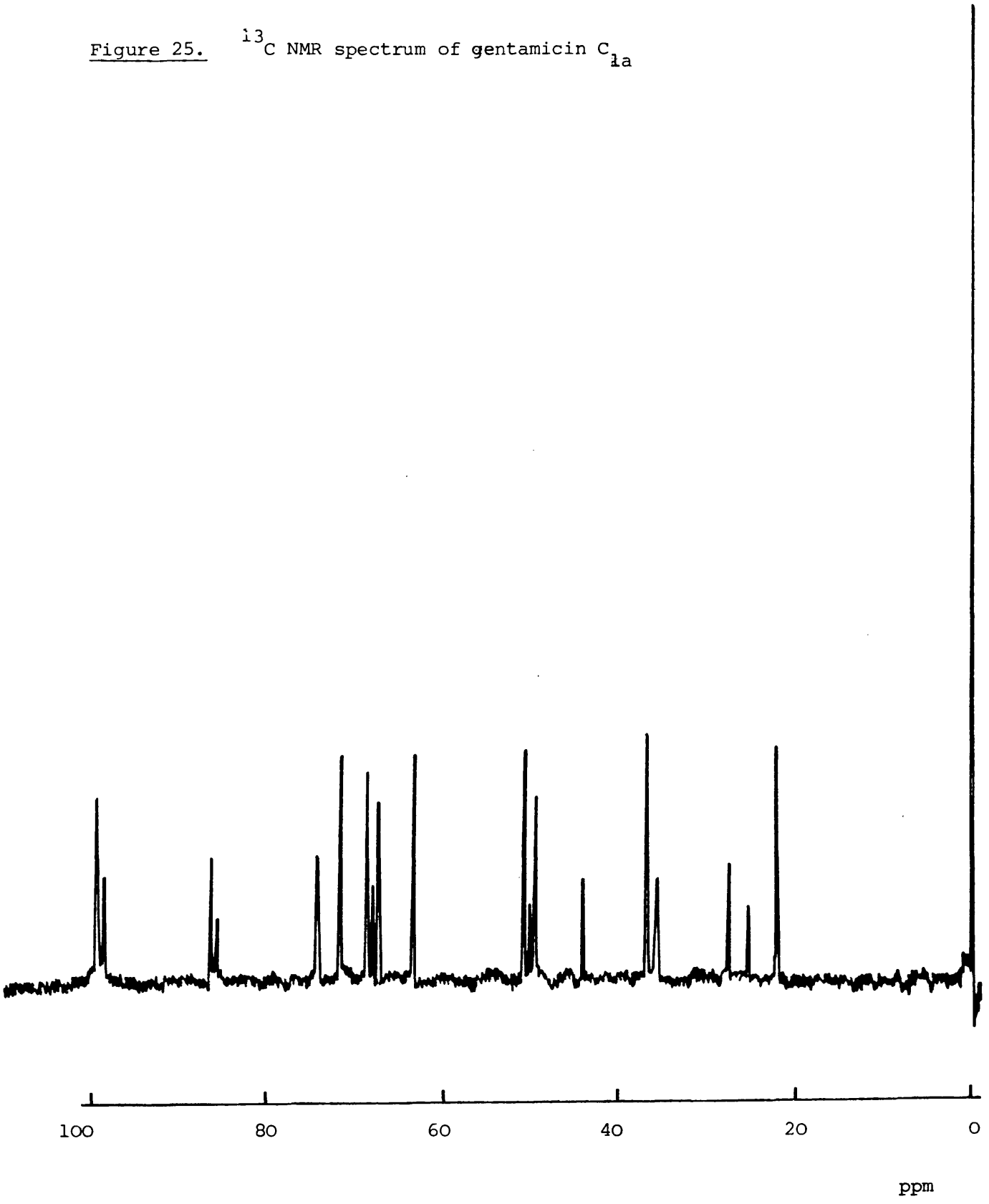
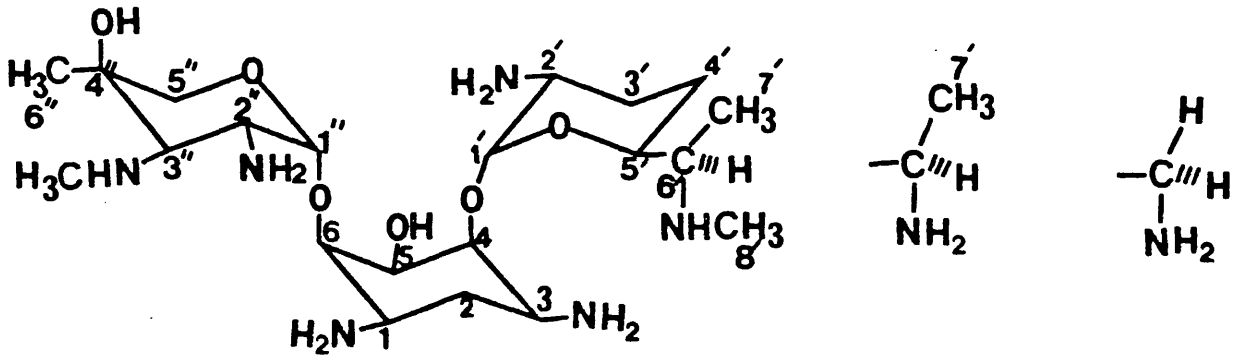


Table 10. $^{13}$C-chemical shifts of gentamicin $C_1$, $C_2$ and $C_{1a}$




| Carbon | Gentamicin $C_1$ | | Gentamicin $C_2$ | | Gentamicin $C_{1a}$ | |
|---|---|---|---|---|---|---|
| | Found | Ref 103 | Found | Ref 103 | Found | Ref 103 |
| 1 | 52.00 | 51.8 | 51.90 | 51.8 | 51.84 | 51.7 |
| 2 | 37.10 | 36.8 | 37.00 | 36.7 | 36.84 | 36.7 |
| 3 | 50.98 | 50.9 | 50.87 | 50.8 | 50.81 | 50.6 |
| 4 | 88.63 | 88.6 | 88.68 | 88.7 | 88.25 | 88.3 |
| 5 | 75.74 | 75.4 | 75.73 | 75.3 | 75.90 | 75.4 |
| 6 | 88.20 | 87.9 | 88.08 | 87.7 | 87.60 | 87.8 |
| 1' | 102.93 | 102.6 | 102.82 | 102.6 | 101.63 | 102.2 |
| 2' | 51.30 | 51.1 | 51.20 | 51.0 | 51.35 | 51.0 |
| 3' | 27.46 | 27.2 | 27.14 | 27.0 | 26.16 | 27.1 |
| 4' | 26.32 | 26.1 | 26.27 | 26.0 | 28.33 | 28.5 |
| 5' | 73.02 | 72.8 | 74.49 | 74.4 | 70.16 | 71.5 |
| 6' | 58.61 | 58.2 | 50.49 | 50.3 | 45.40 | 46.1 |
| 7' | 15.00 | 15.0 | 18.79 | 19.0 | | |
| 8' | 33.80 | 33.7 | | | | |
| 1" | 101.68 | 101.4 | 101.57 | 101.3 | 100.82 | 101.3 |
| 2" | 70.64 | 70.3 | 70.59 | 70.2 | 69.61 | 70.2 |
| 3" | 64.73 | 64.5 | 64.68 | 64.4 | 64.84 | 64.4 |
| 4" | 73.68 | 73.3 | 73.62 | 73.2 | 73.24 | 73.3 |
| 5" | 69.07 | 68.7 | 69.01 | 68.7 | 69.01 | 68.7 |
| 6" | 23.07 | 22.9 | 22.97 | 22.8 | 22.91 | 23.0 |
| 7" | 38.24 | 38.1 | 38.14 | 38.1 | 37.87 | 38.0 |

Figure 26. $^{13}C$ NMR spectrum of gentamicin base mixture.

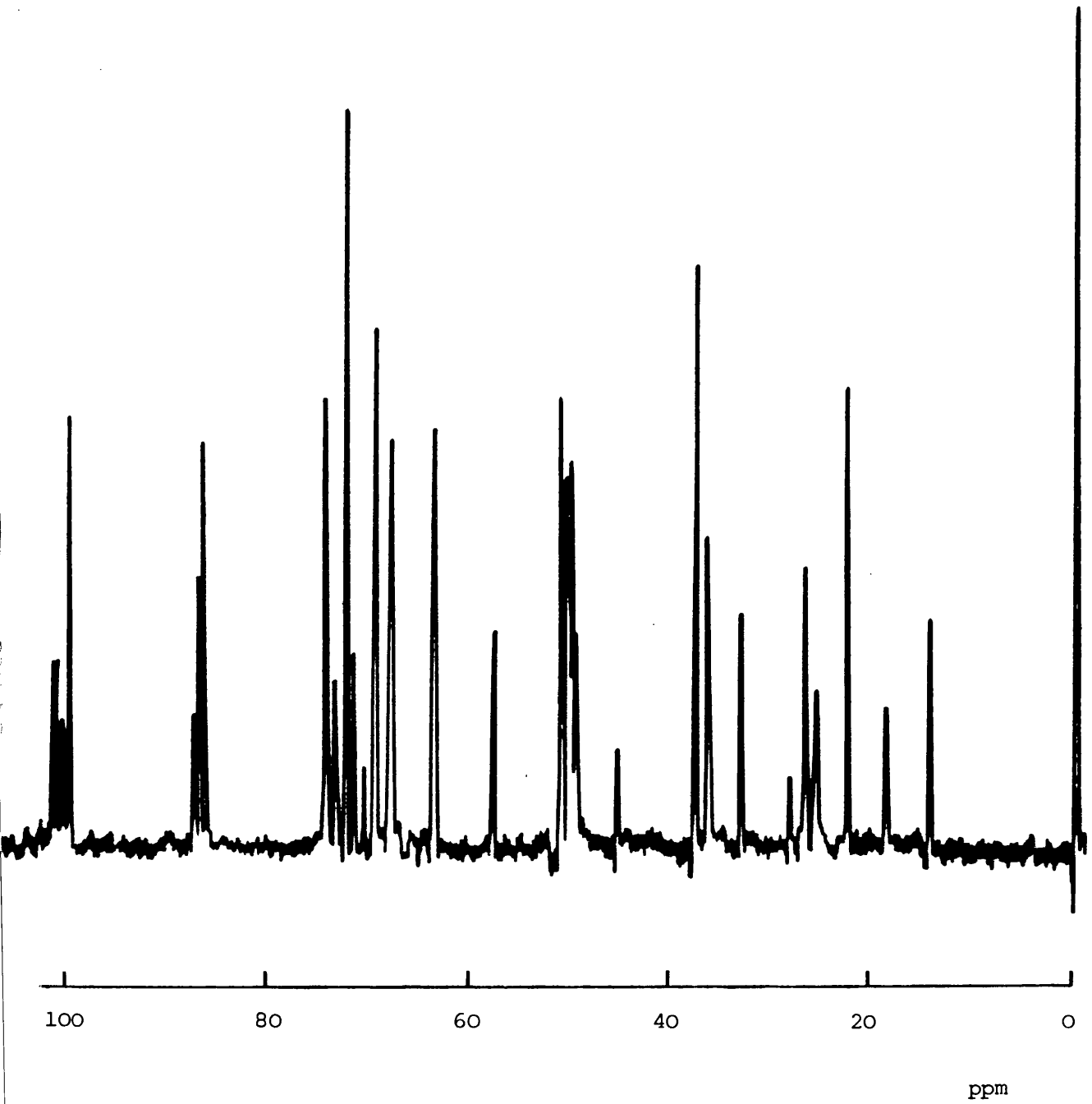


Figure 26. (contd.) $^{13}C$ NMR spectrum of gentamicin sulphate mixture.

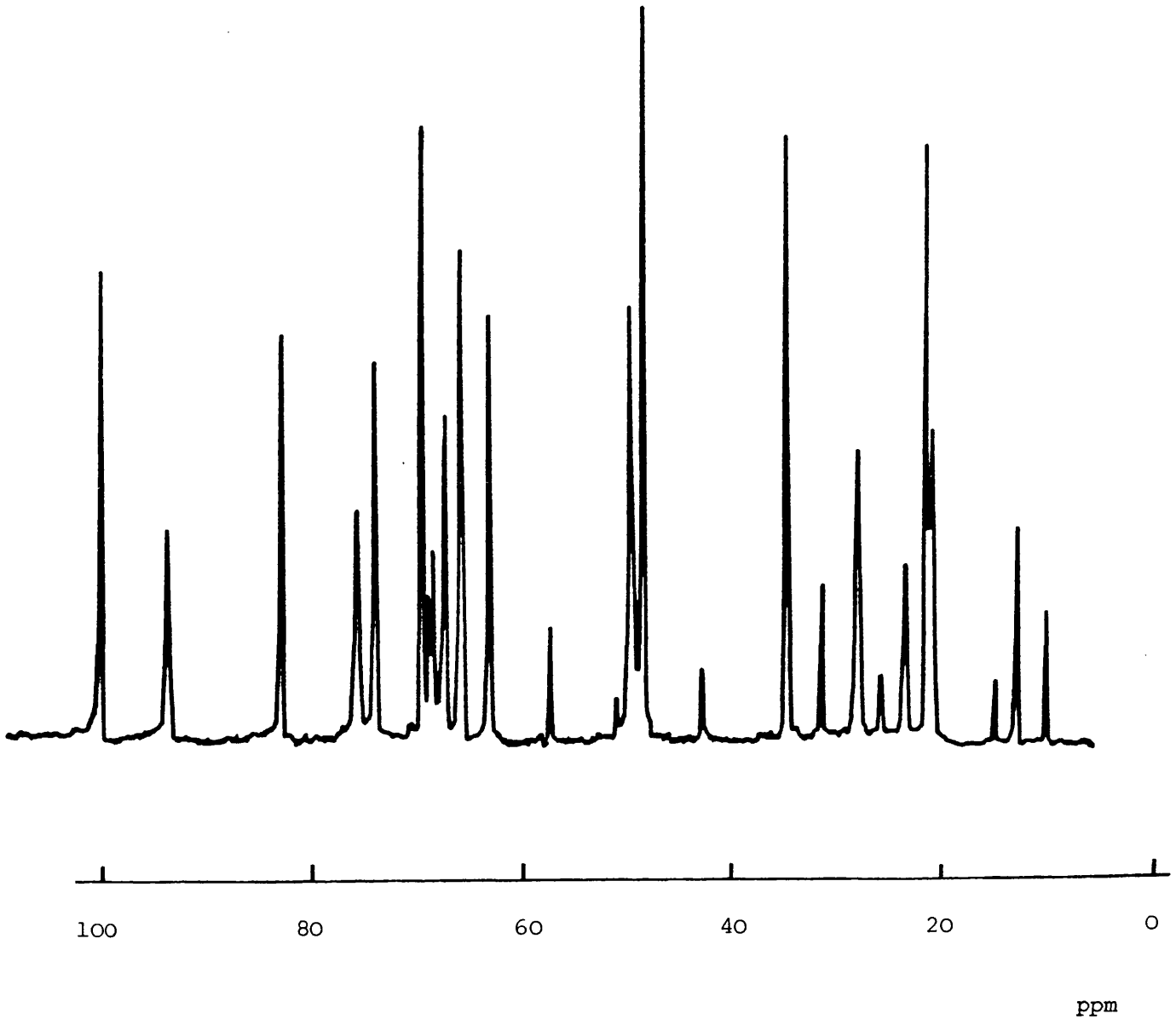
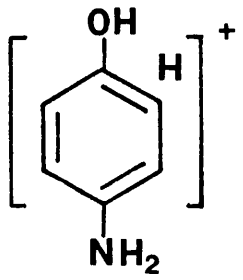


Table 11

| CARBON | CHEMICAL SHIFT (ppm) | |
|---|---|---|
| | FREE BASE | SULPHATE |
| 1 | 51.95 | 50.79 |
| 2 | 37.11 | 35.60 |
| 3 | 50.92 | 49.57 |
| 4 | 88.84($C_2$), 88.35 ($C_1$&$C_{1a}$) | 84.47 |
| 5 | 75.80 | 76.61 |
| 6 | 88.20 | 84.47 |
| 1' | 102.98($C_1$),102.71($C_2$), 102.44($C_{1a}$) | 95.35 |
| 2' | 51.25 | 49.57 |
| 3' | 27.25 | 24.39 |
| 4' | 26.22 ($C_1$&$C_2$), 28.76($C_{1a}$) | 28.62 |
| 5' | 74.70($C_2$),72.75($C_1$), 71.72($C_{1a}$) | 74.97($C_1$),69.87($C_2$), 69.37($C_{1a}$) |
| 6' | 58.67($C_1$),50.54($C_2$), 46.26($C_{1a}$) | 58.24($C_1$), 49.57($C_2$), 43.62($C_{1a}$) |
| 7' | 19.01($C_2$), 14.68($C_1$) | 13.12 ($C_2$), 10.14($C_1$) |
| 8' | 33.64($C_1$) | 32.03 |
| 1" | 101.68 | 102.01 |
| 2" | 70.64 | 64.42 |
| 3" | 64.73 | 64.73 |
| 4" | 73.68 | 70.81 |
| 5" | 69.07 | 69.02 |
| 6" | 23.02 | 21.90 |
| 7" | 38.24 | 35.60 |

2) Compound $CX_2$

Figure 27 shows the isobutane chemical ionisation spectrum of compound $CX_2$. The $[M + H]^+$ quasimolecular ion appears at m/e 322. The base peak at m/e 163 suggests the presence of a 2-deoxystreptamine moiety whilst the peak at m/e 110 might reflect loss of $2H_2O$ and $NH_3$ from this to yield an aromatic ion such as




The garosamine unit gives a peak at m/e 160 and the peak at m/e 129 may be due to loss of methylamine from this.

The $^1$H-NMR spectrum of $CX_2$ (Figure 28) supports a bicylic deoxystreptamine-garosamine ring structure from $CX_2$. Prominent singlets at $\delta$1.3 and $\delta$2.85 may be respectively the C-$CH_3$ and NH-$CH_3$ of garosamine. These peaks have similar chemical shifts to those observed in gentamicin sulphate which suggests that this sample is ionised; perhaps as its carbonate salt.

Only one anomeric proton (1") is present as a doublet at $\delta$5.00. Other ring proton resonances between $\delta$2.00 and $\delta$5.00 resemble those in the spectra of gentamicin C.

Table 12 shows possible assignments based on the corresponding protons of gentamicin C.

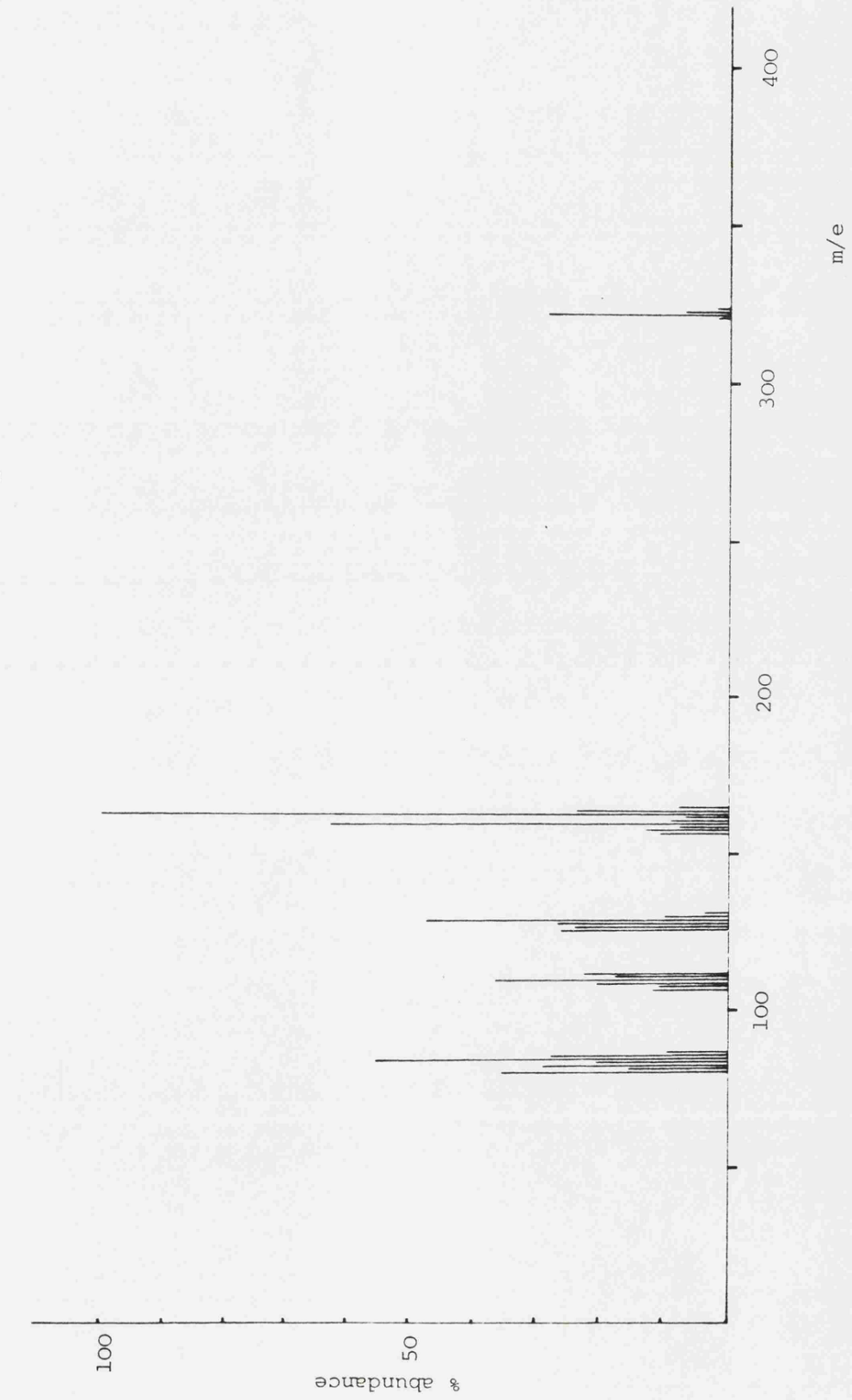



Figure 27. Chemical ionisation mass spectrum of compound $CX_2$

Figure 28. PMR Spectrum of compound $CX_2$

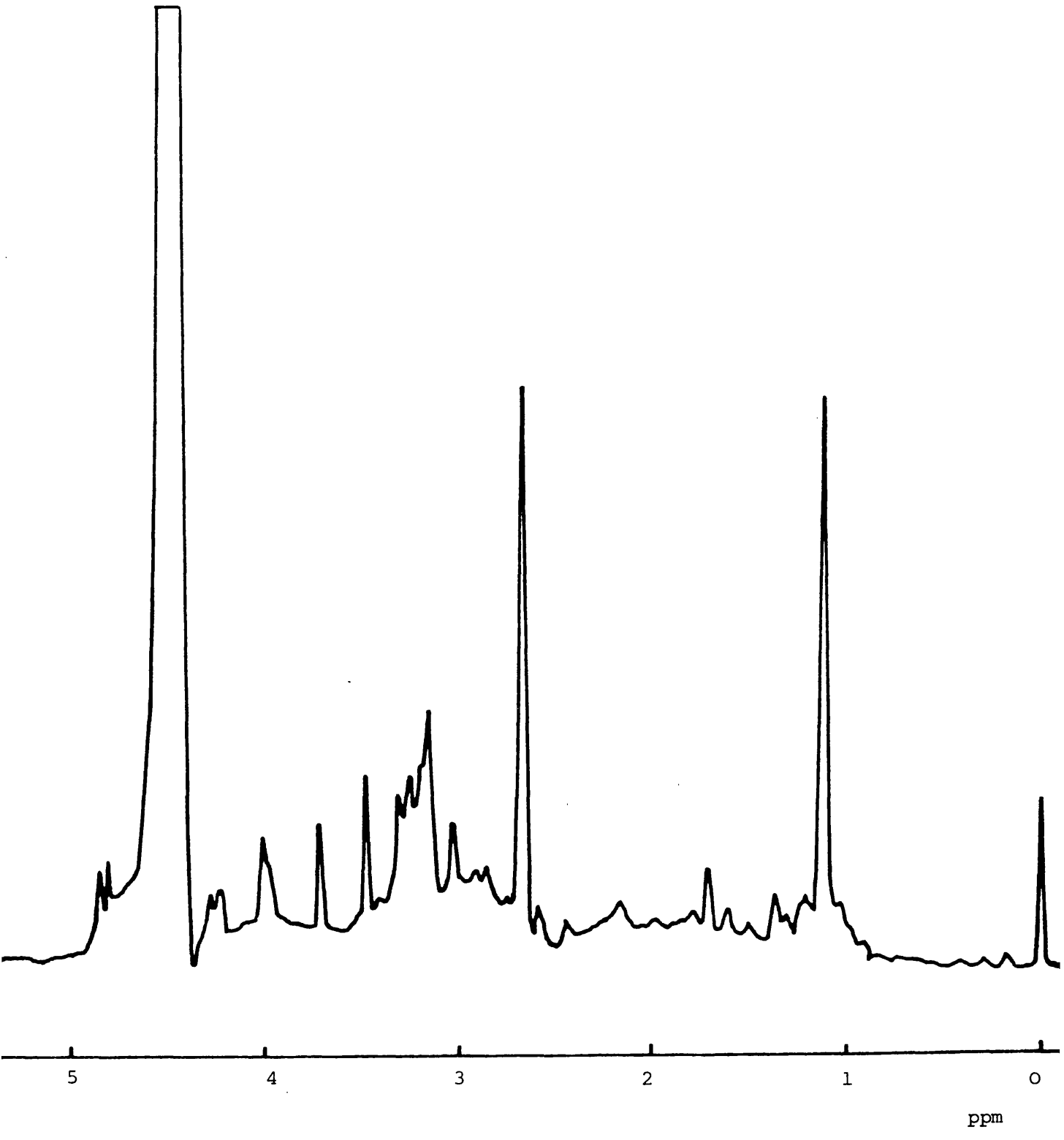


Table 12.

| Proton | Chemical shifts (ppm) |
|---|---|
| $N-CH_3$ | 2.85 |
| $C-CH_3$ | 1.30 |
| 1" | 5.00 |
| 2" eq | 4.29 |
| 5" eq | 4.08 |
| 5" ax | 3.38 |

Figure 29 shows a $^{13}C$ spectrum of compound $CX_2$ and Figure 30 shows a possible structure and the assignment of $^{13}C$ chemical shifts compared with literature values (187 - 196) for 1-methyl garosamine and 2-deoxystreptamine (103).

The assignment of identical chemical shifts to carbon atoms 1 and 3 and to 4 and 6 suggests that the 2-deoxystreptamine moiety is symmetrical and that the glycosidic link may be between the garosamine and the 5 position of the 2-deoxystreptamine instead of to the 6 position of 2-deoxystreptamine ring as in the major gentamicin C components.

Figure 29. $^{13}C$ spectrum of compound $CX_2$

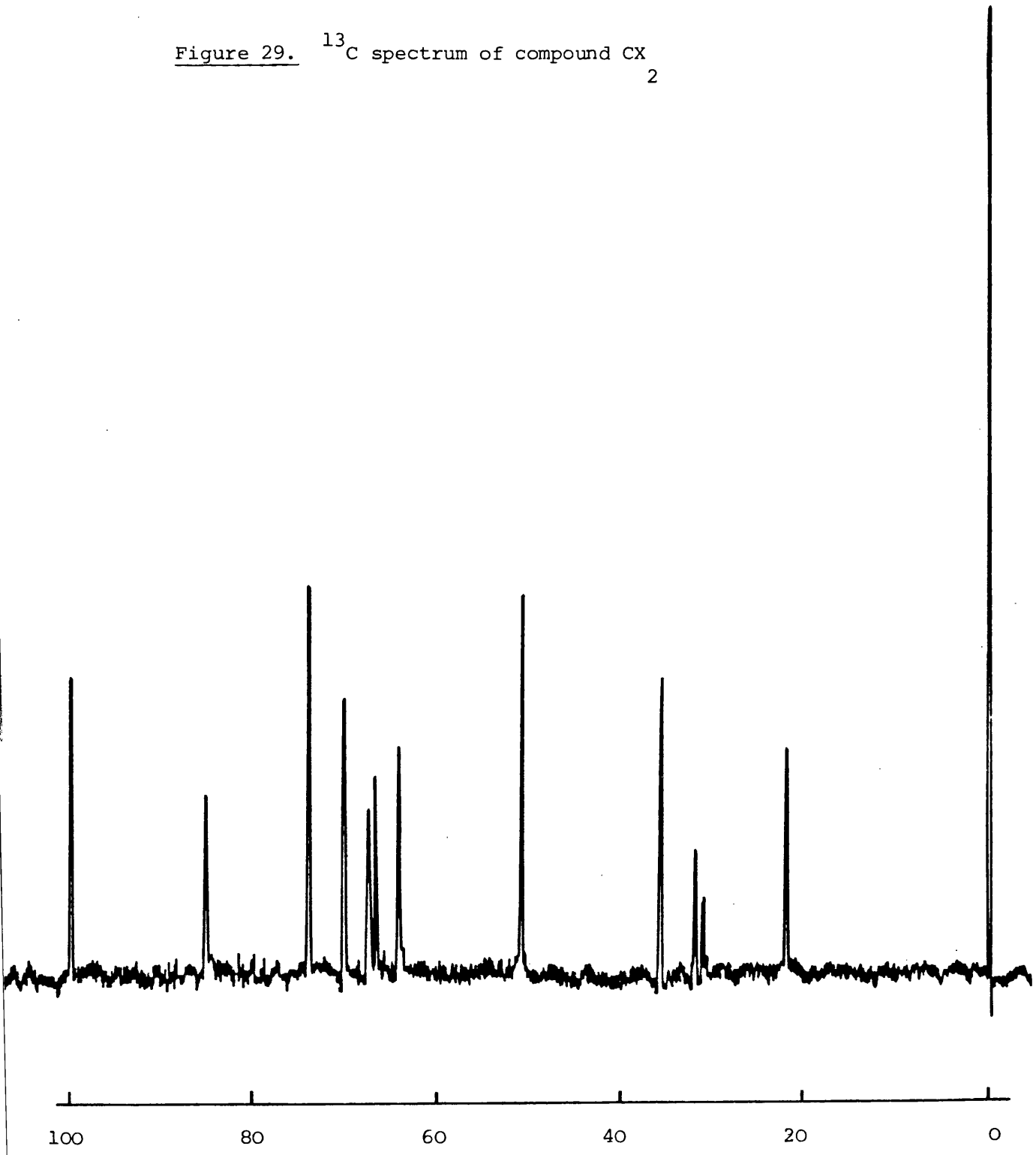
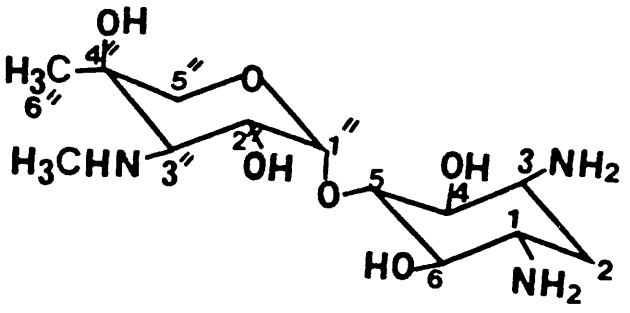


Figure 30.




| Carbon | Chemical shift (ppm) | | |
|---|---|---|---|
| | $CX_2$ | 1-methyl garosamine | 2-deoxystreptamine |
| 1 | 51.68 | | 51.6 |
| 2 | 32.50 | | 37.0 |
| 3 | 51.68 | | 51.6 |
| 4 | 75.25 | | 78.5 |
| 5 | 86.46 | | 76.6 |
| 6 | 75.25 | | 78.5 |
| 1" | 101.52 | 100.6 | |
| 2" | 68.58 | 70.1 | |
| 3" | 65.33 | 64.6 | |
| 4" | 71.40 | 73.4 | |
| 5" | 67.99 | 68.0 | |
| 6" | 22.37 | 22.5 | |
| $NH-CH_3$ | 36.30 | 38.0 | |

3) Compound $CX_6$

Figure 31 shows the chemical ionisation mass spectrum of compound $CX_6$. The $[M + H]^+$ quasimolecular ion and the fragmentation patterns are similar to those of compound $CX_2$.

The $^1H$ NMR spectrum of $CX_6$ (Figure 32) is also similar to that of compound $CX_2$.

The $^{13}C$ NMR spectrum of $CX_6$ (Figure 33) is much more complex than that of $CX_2$ and the sample may have been a mixture.

Berdy et al. (166) isolated one compound of M.W. 321 from the crude fermentation broth and identified it as garamine (Figure 31). Of the two compounds with M.W. 321 isolated in this study the $^{13}C$ NMR spectrum of $CX_2$ suggests that the pseudodisaccharide may have a 1", 5 glycosidic bond. Thus $CX_6$ may be garamine. The $^{13}C$ NMR spectrum of $CX_6$ does contain peaks consistent with this structure (Figure 34) though this leaves prominent peaks with chemical shifts of 44.04 and 26.78 ppm unexplained.

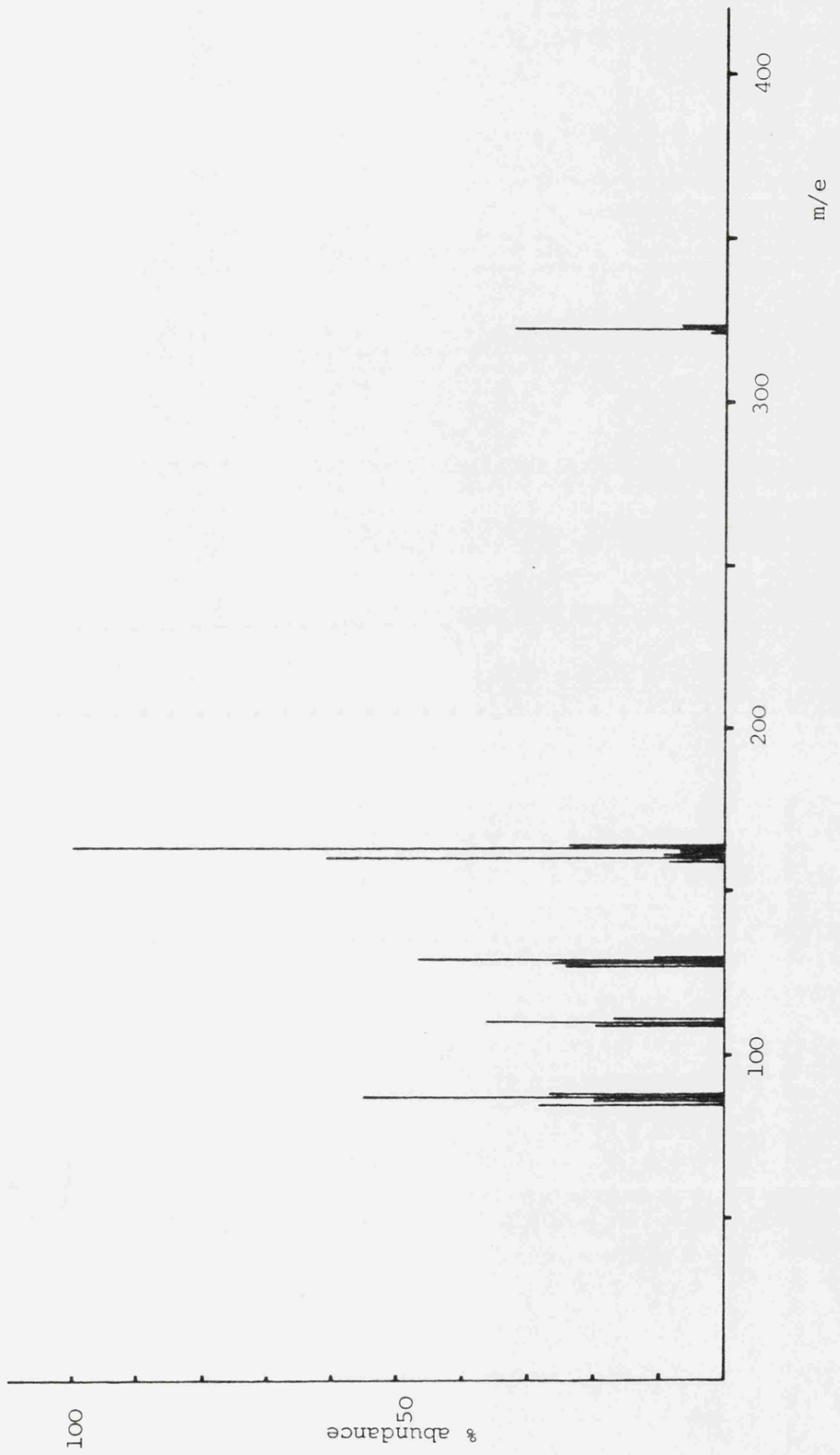



Figure 31. Chemical ionisation mass spectrum of compound $CX_6$

Figure 32. PMR spectrum of compound $CX_6$

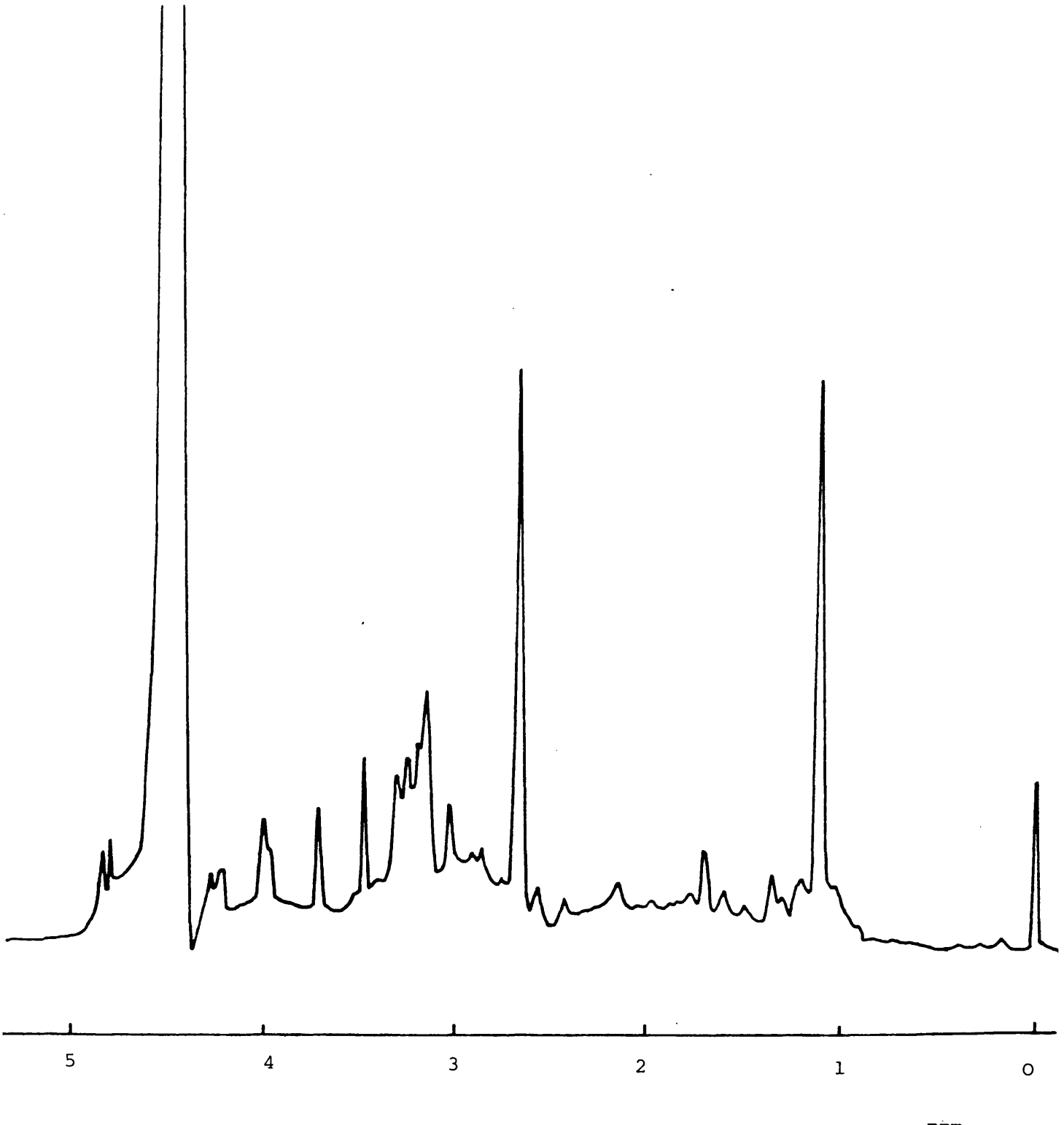


Figure 33. $^{13}C$ NMR spectrum of compound $CX_6$

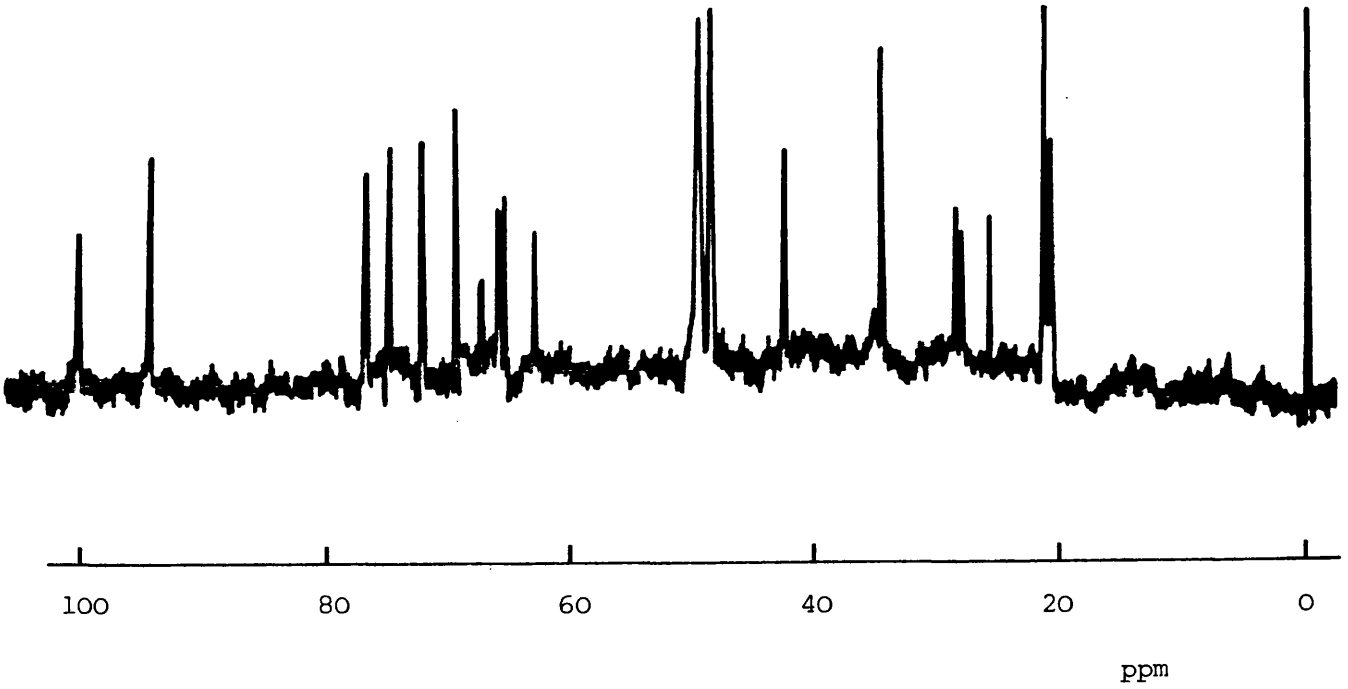
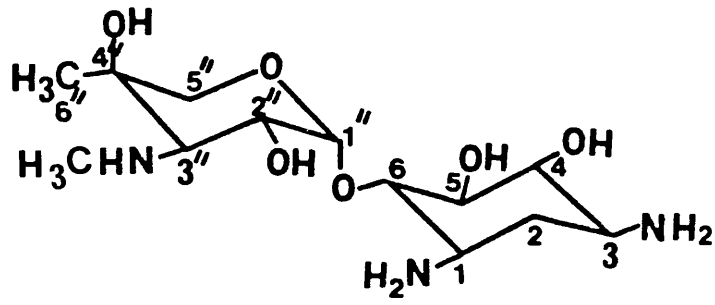


Figure 34. A possible structure and the assignment of $^{13}C$ chemical shifts compared with the literature values.




| Carbon | Chemical shift (ppm) |
|---|---|
| 1 | 50.16 |
| 2 | 30.12 |
| 3 | 50.76 |
| 4 | 75.99 |
| 5 | 78.16 |
| 6 | 96.37 |
| 1" | 102.28 |
| 2" | 70.77 |
| 3" | 64.20 |
| 4" | 73.38 |
| 5" | 66.85 |
| 6" | 22.42 |
| $NH-CH_3$ | 36.02 |

4) Compound $CX_3$

Figures 35 and 36 show electron impact and chemical ionisation mass spectra of compound $CX_3$. They show similar fragmentation patterns.

The peak at m/e 445 is assumed by analogy with gentamicin C to be a $[M + H]^+$ quasimolecular ion, implying a molecular weight of 444. Peaks at 322, 163 and 160 corresponding to garamine, 2-deoxytreptamine and garosamine moieties respectively suggest that this compound differs from the major components of gentamicin in its A (purpurosamine) ring. In their study of minor components from the fermentation broth, Berdy et al.(166) isolated a compound of M.W. 444 to which they allocated to structure in Figure 37 but gave no further details of its spectroscopic characteristics. The peak at m/e 350 might arise from such a structure as outlined in Figure 37.

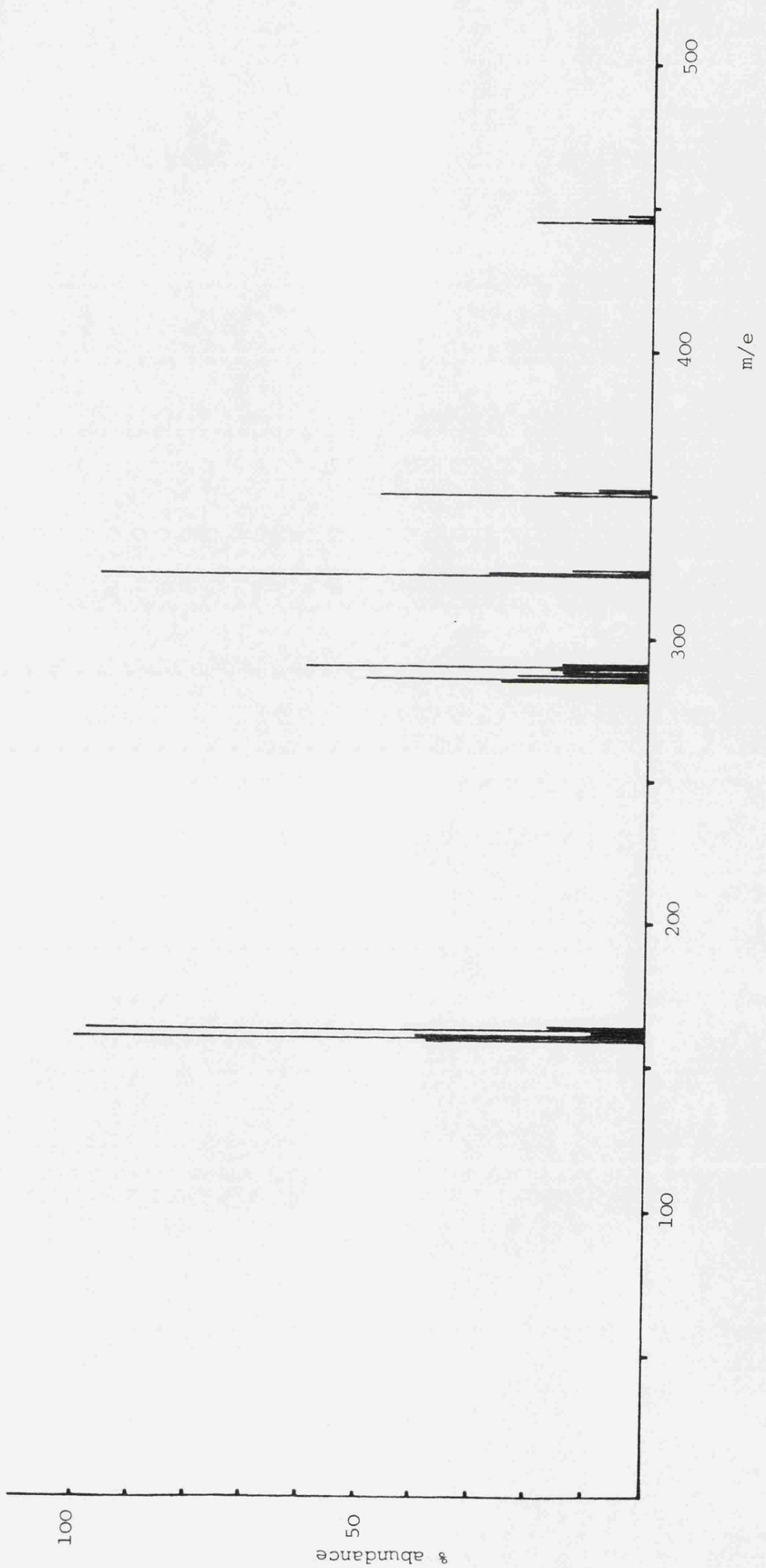



Figure 35. Electron impact mass spectrum of compound $CX_3$

Figure 36. Chemical ionisation mass spectrum of compound $CX_3$

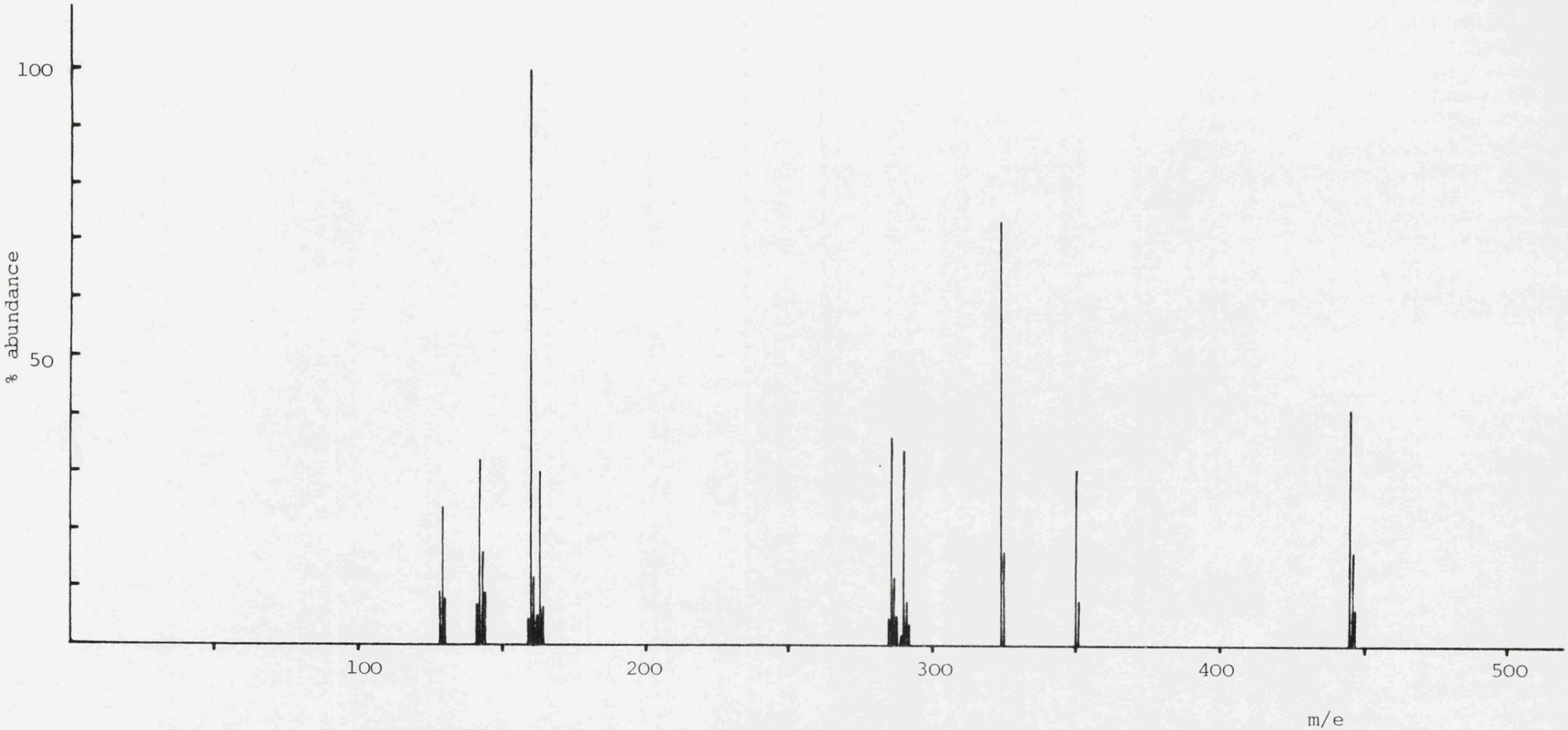
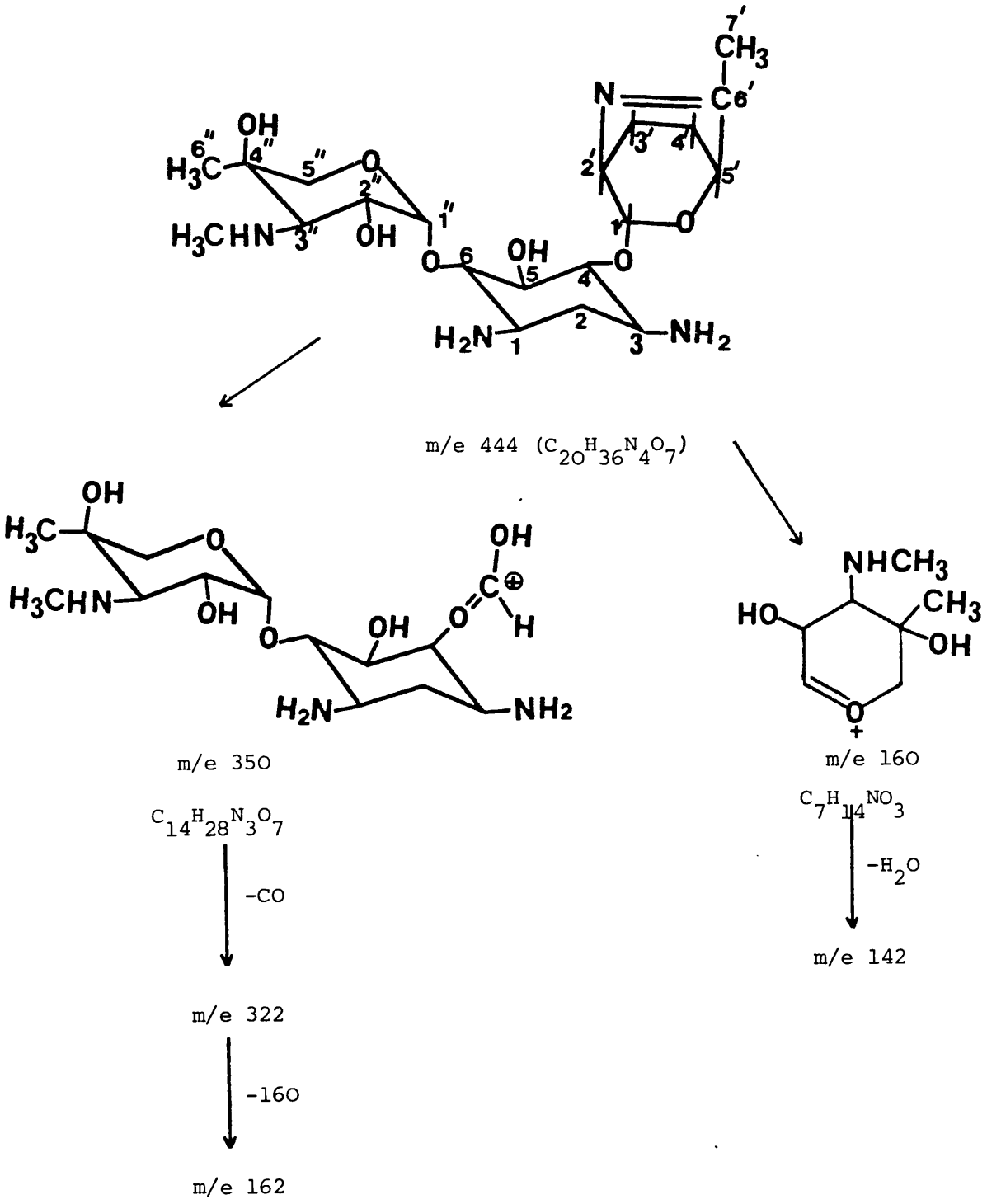


Figure 37. A possible structure and mass spectral fragmentation of Compound $CX_3$.



In the $^{1}$H-NMR spectrum (Figure 38) the peak at δ2.21 may represent the C-7'methyl group of the compound whilst peaks at δ1.29 and δ2.73 may be due to the C-methyl and N-methyl groups of garosamine. Other resonances may be allocated as in Table 13.

| Proton | Chemical shift (ppm) |
|---|---|
| 1" | 5.31 |
| 2" | 4.06 |
| 5" eq | 4.30 |
| 1' | 5.54 |
| 5' | 3.40 |

Figure 38. PMR Spectrum of compound $CX_3$

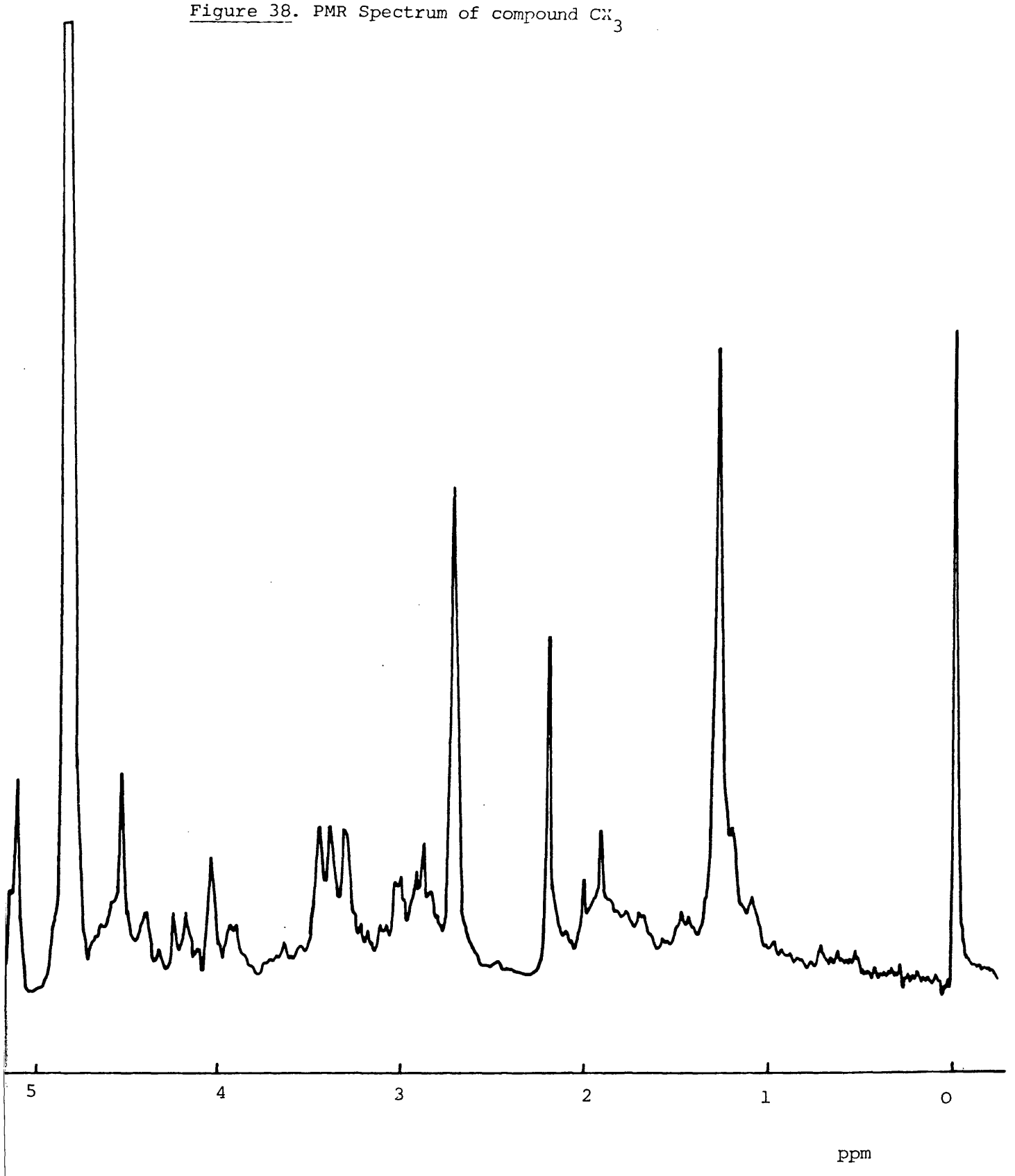
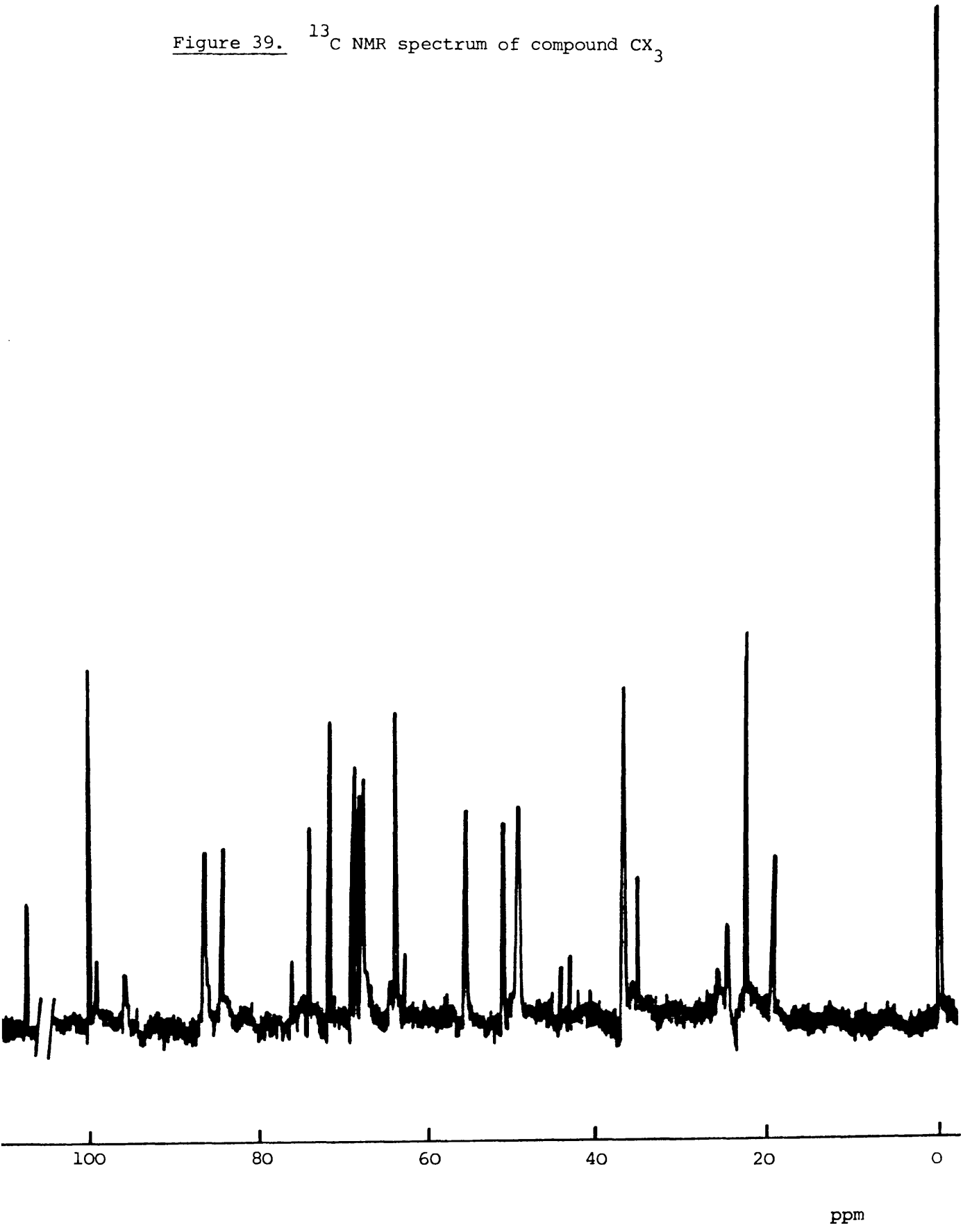
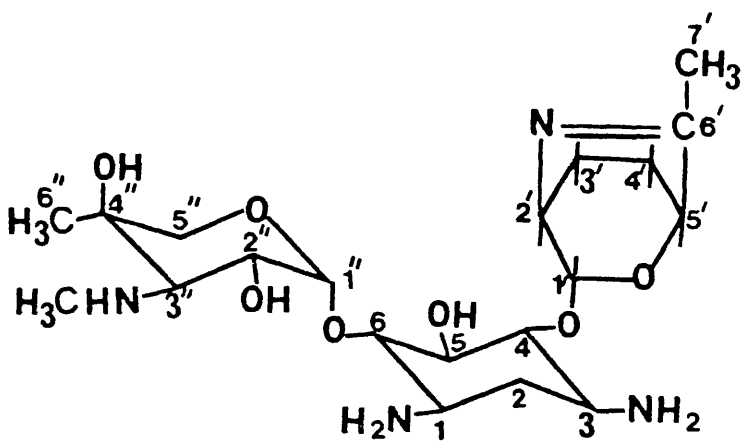


Figure 39. $^{13}C$ NMR spectrum of compound $CX_3$



The $^{13}C$ NMR spectrum shown in Figure 39 also seems to be consistent with this possible structure shown in Figure 40.

Figure 40

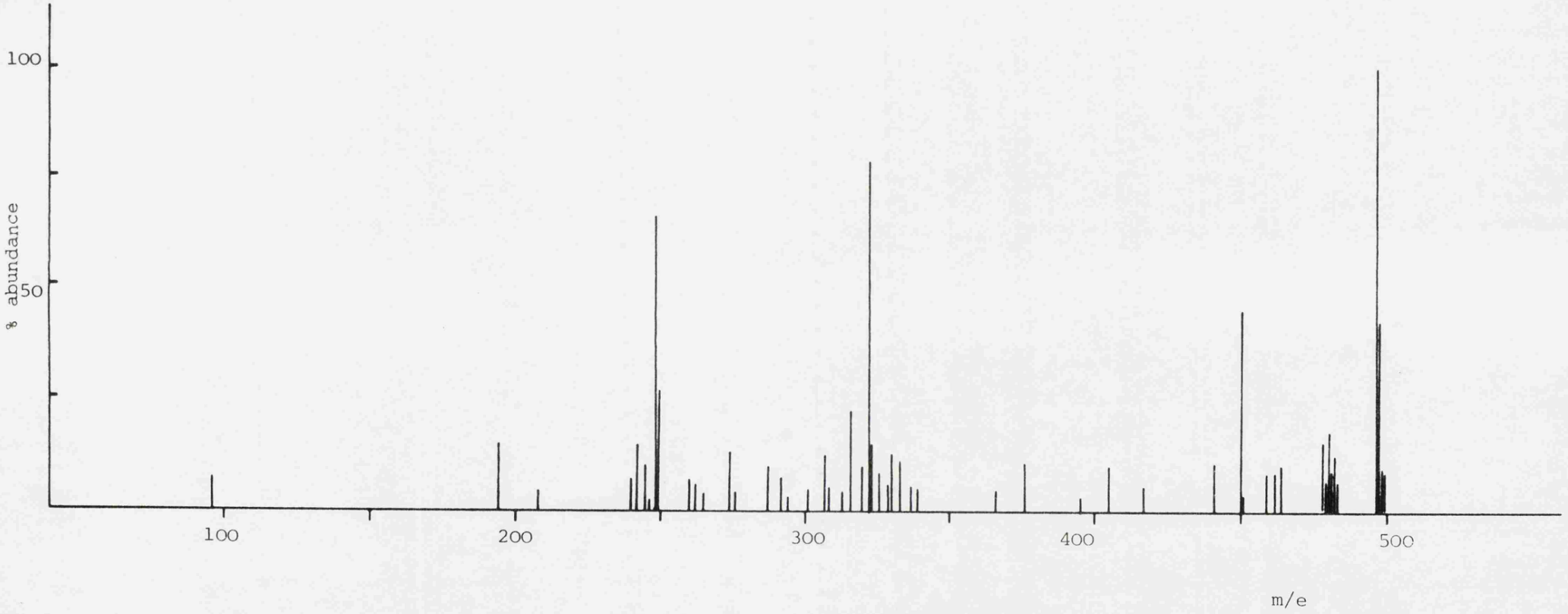



| Carbon | Chemical shift (ppm) |
|---|---|
| 1 | 52.00 |
| 2 | 35.81 |
| 3 | 50.38 |
| 4 | 87.76 |
| 5 | 75.25 |
| 6 | 85.54 |
| 1' | 97.35 |
| 2' | 56.67 |
| 3' | 26.16 |
| 4' | 25.06 |
| 5' | 69.01 |
| 6' | 183.00 |
| 7' | 19.56 |
| 1" | 101.69 |
| 2" | 69.61 |
| 3" | 65.00 |
| 4" | 72.76 |
| 5" | 68.80 |
| 6" | 22.75 |
| $NH-CH_3$ | 37.43 |

5) Compound $CX_4$

The field desorption mass spectrum (Figure 41) of this compound gave an ion of m/e 496 and the chemical ionisation spectrum (Figure 42) one at m/e 495. This may imply that m/e 495 is a true $M^+$ and that m/e 496 is $[M + H]^+$ . The electron impact mass spectrum showed no peak higher than m/e 258 suggesting that this compound is particularly unstable. This conclusion is supported by the appearance of fragment ions at m/e 450, 322 and 249 in the field desorption mass spectrum and by the relatively low m/e 496 ion in the chemical ionisation mass spectrum. In this latter spectrum the ions at m/e 322, 163 and 160 suggest garamine, 2-deoxystreptamine and garosamine moieties respectively. The peak at m/e 336 may represent the A and B rings. Berdy *et al.* (116) isolated a compound with molecular weight 495 from the fermentation broth and assigned the structure shown in Figure 43, though they presented no further evidence.

Figure 41. Field Desorption mass speetrum of compound $CX_4$



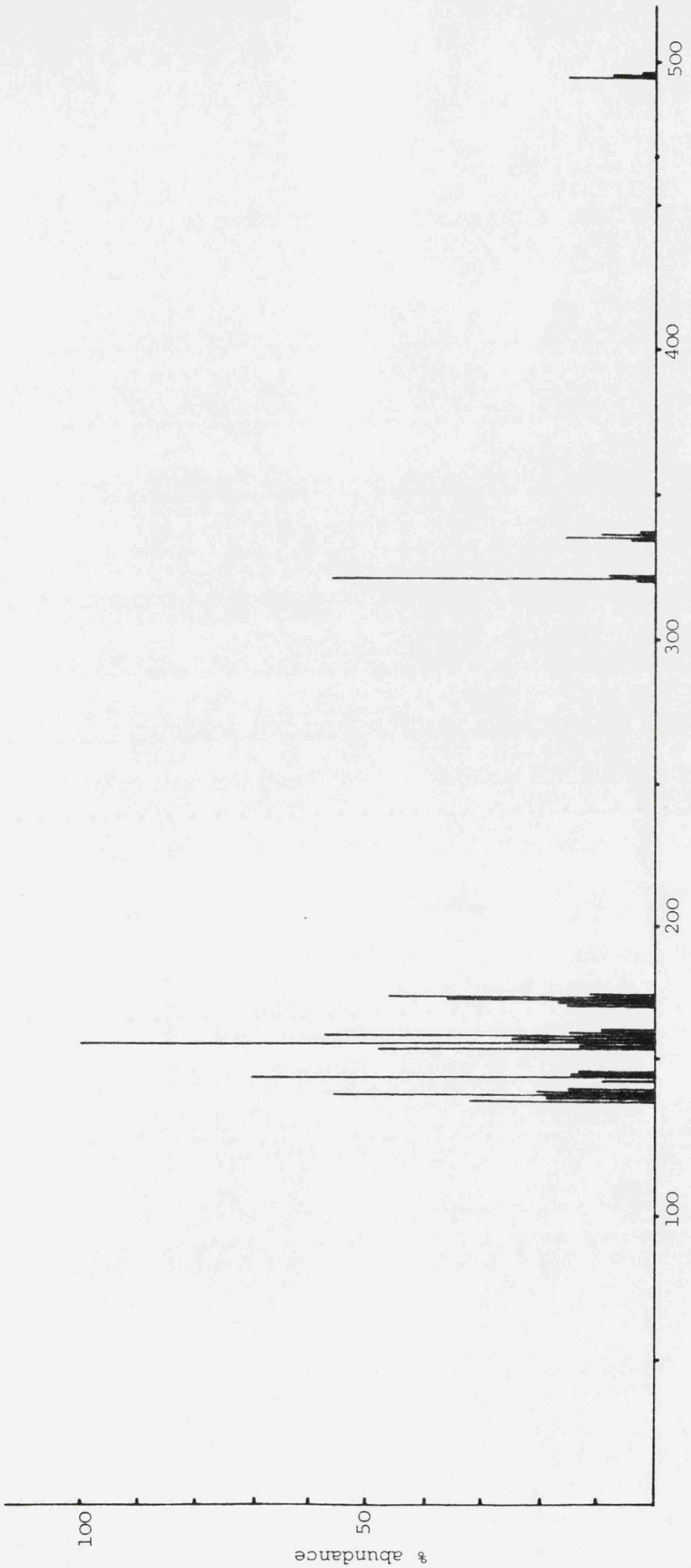
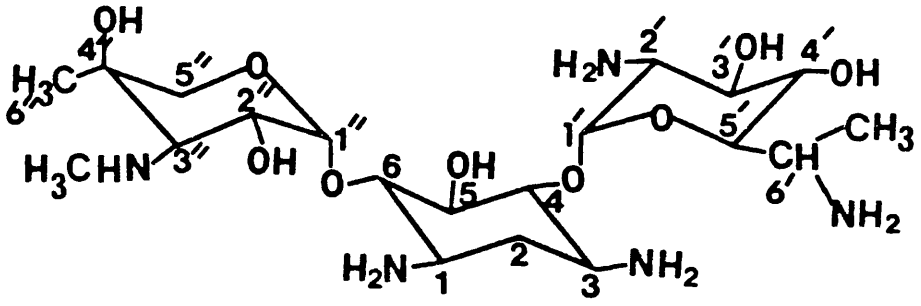



Figure 42. Chemical ionisation mass spectrum of compound $CX_4$

Figure 43. The structure of compound 13 (M.W. 495) isolated by Berdy et al. (166)




The $^{1}$H-NMR spectrum (Figure 44) showed prominent peaks at δ2.97 and δ1.37 which may be the -NH-$CH_3$ and C-$CH_3$ respectively of garosamine. A doublet centred at δ1.20 may be a secondary C-methyl group as shown in the structure. The compound appears to be ionised since the resonances of the anomeric protons appear at δ6.11 (1') and at δ5.38.

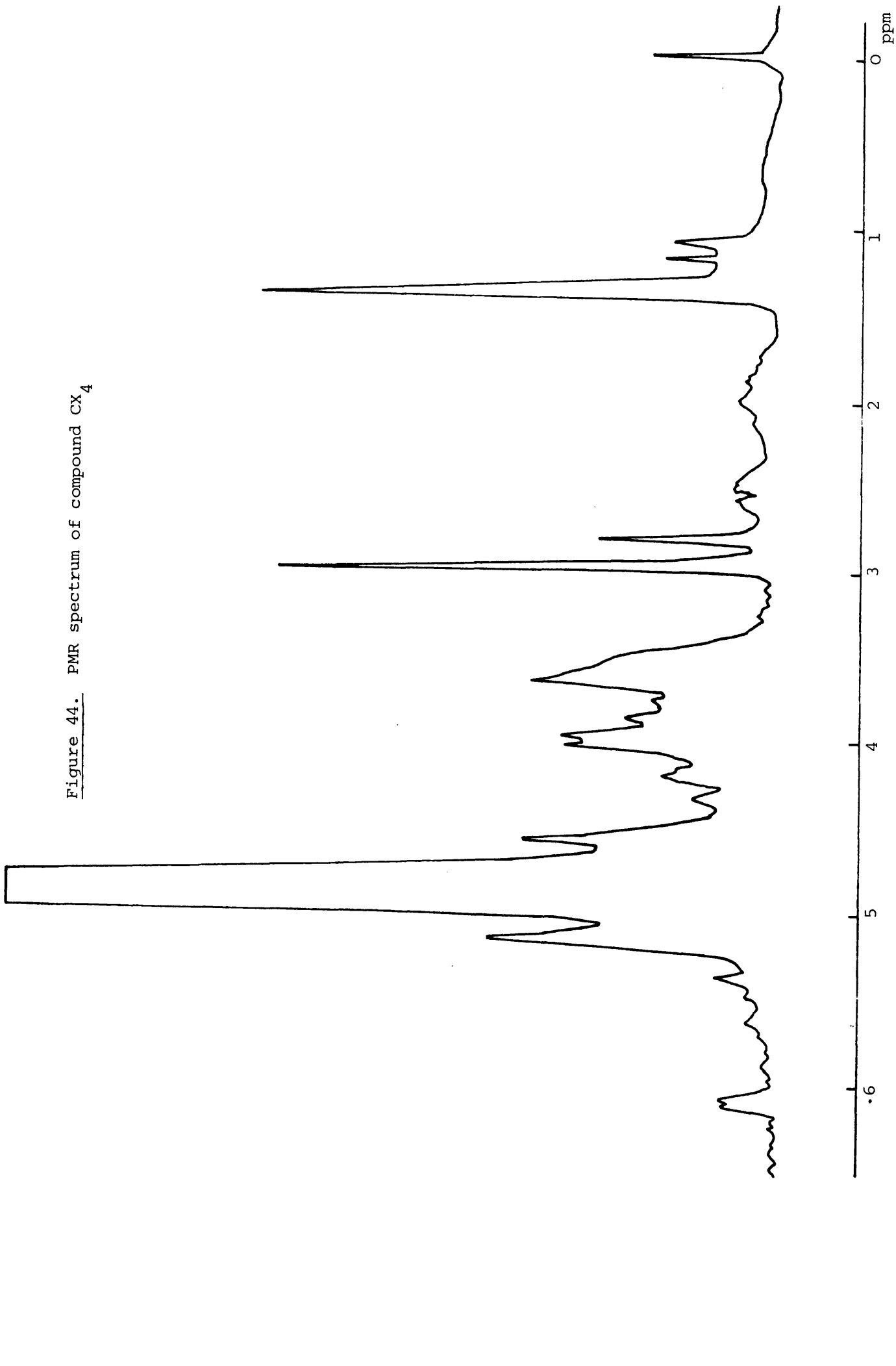



Figure 44. PMR spectrum of compound $CX_4$

Figure 45. $^{13}C$ NMR spectrum of compound $CX_4$

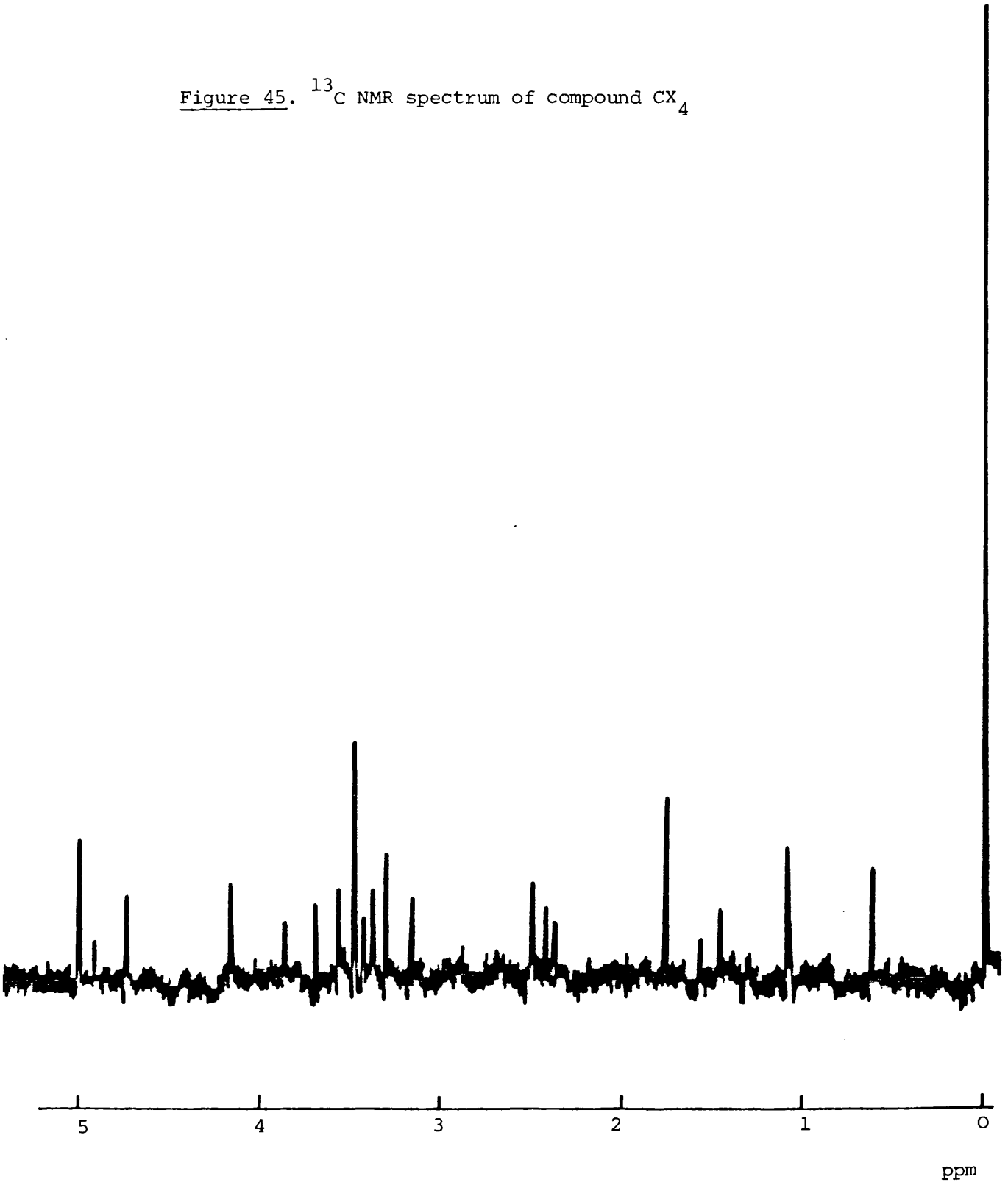
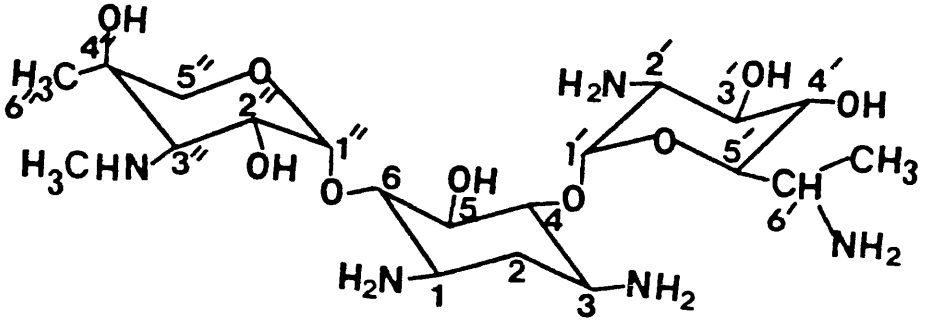


Figure 45 shows $^{13}C$ spectrum of compound $CX_4$ and Figure 46 shows its assigned chemical shifts.

Figure 46.




| Carbon | Chemical shifts (ppm) |
|---|---|
| 1 | 49.80 |
| 2 | 30.12 |
| 3 | 48.86 |
| 4 | 79.14 |
| 5 | 73.08 |
| 6 | 85.27 |
| 1' | 96.86 |
| 2' | 51.24 |
| 3' | 70.04 |
| 4' | 70.11 |
| 5' | 73.08 |
| 6' | 49.80 |
| 7' | 12.83 |
| 1" | 102.44 |
| 2" | 67.66 |
| 3" | 64.75 |
| 4" | 71.34 |
| 5" | 67.66 |
| 6" | 22.33 |
| NH-$CH_3$ | 36.08 |

These chemical shifts are comparable with those of the salt form of gentamicin A (195) suggesting that the compound is in an ionised form.

6) Compound $CX_1$

This compound was recovered in very low yield, frequently contaminated with gentamicin $C_1$. The chemical ionisation mass spectrum (Figure 47) gave a peak at m/e 489. No useful NMR data could be obtained.

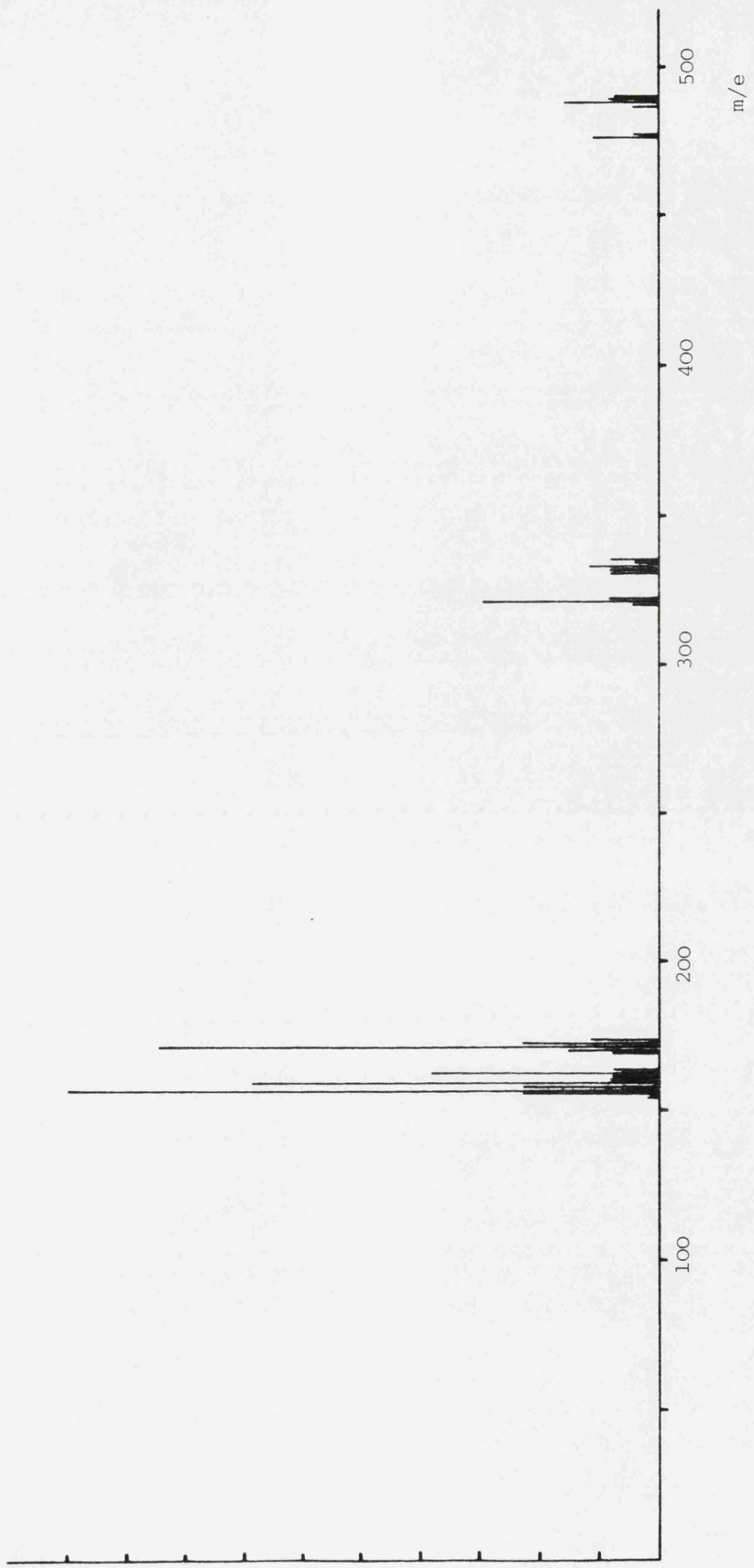



Figure 47. Chemical ionisation mass spectrum of compound $CX_1$

7) Compound $CX_5$

Figures 48 and 49 show chemical ionisation and field desorption spectra of this compound. They suggest that $CX_5$ may be a mixture of compounds giving $[M + H]^+$ peaks of 474, 460 and 446 and differing in their degree of methylation plus a compound with $[M + H]^+$ of 486. It is noteworthy that there is no peak at m/e 322 in the chemical ionisation spectrum suggesting that the bicyclic unit of this compound is different from that of the compounds previously described. The peak at m/e 160 in the chemical ionisation spectrum suggests the presence of a garosamine unit. If so the 2-deoxystreptamine ring is absent. The peak at m/e 157 suggests an 'A' ring of the same type as gentamicin $C_1$.

Insufficient material was available for $^{13}C$ spectrum. The $^1H$-NMR spectrum (Figure 50) is rather 'noisy' but shows peaks corresponding to the C-methyl of gentamicin and three peaks in the N-methyl region.

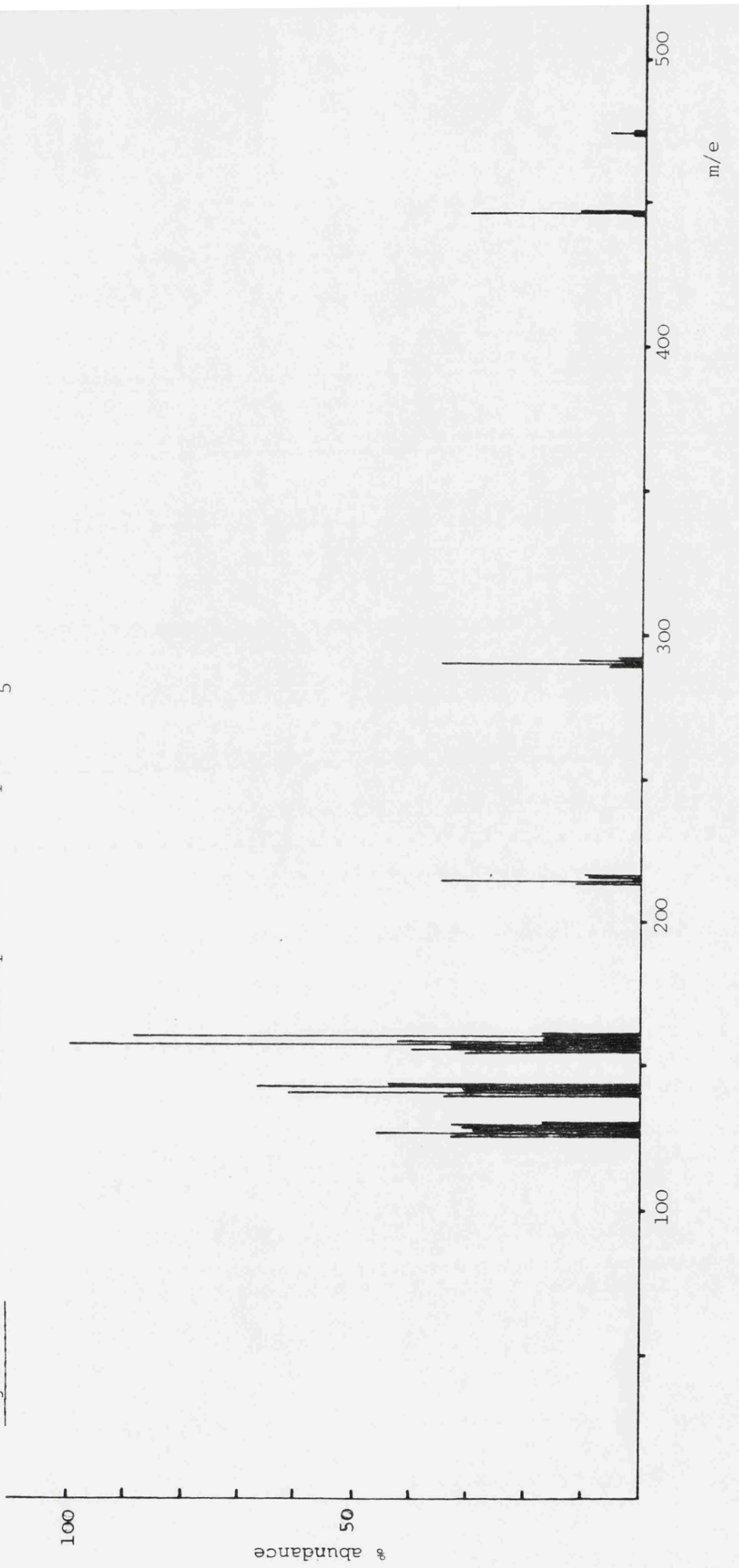



Figure 48. Chemical ionisation mass spectrum of compound $CX_5$

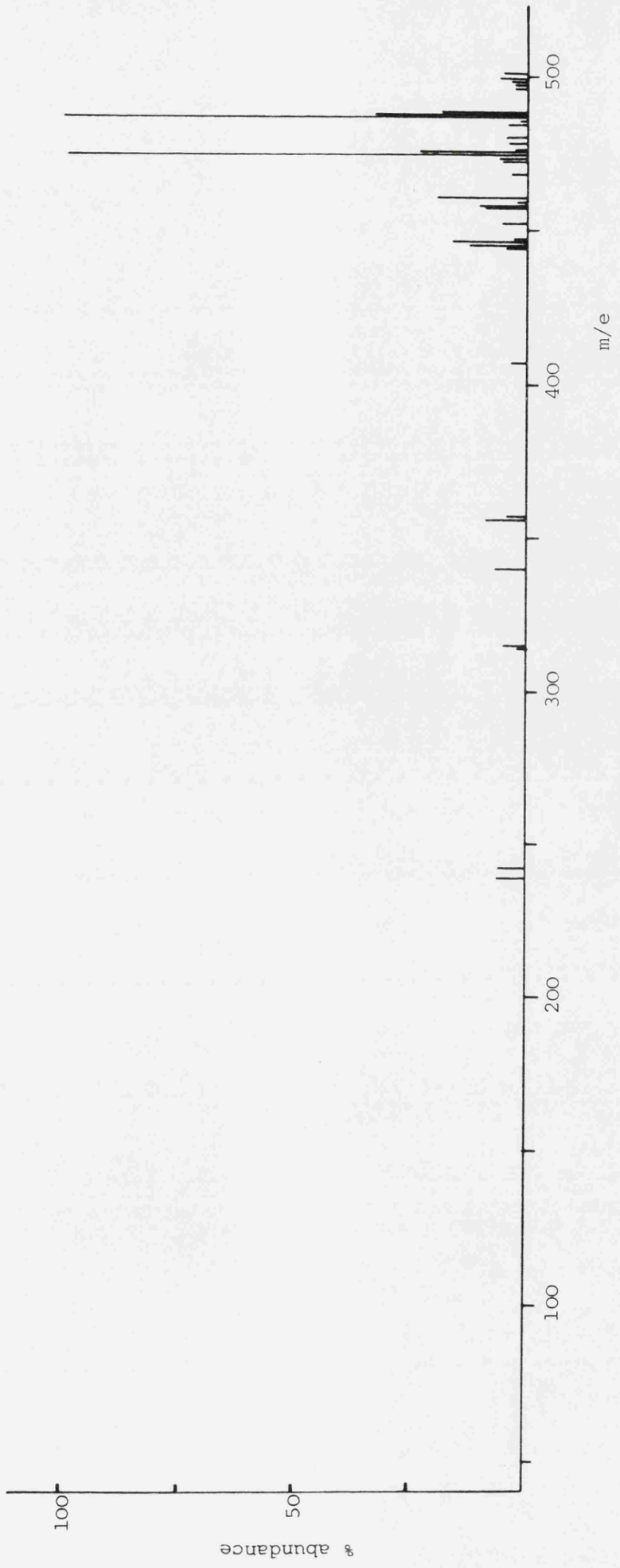
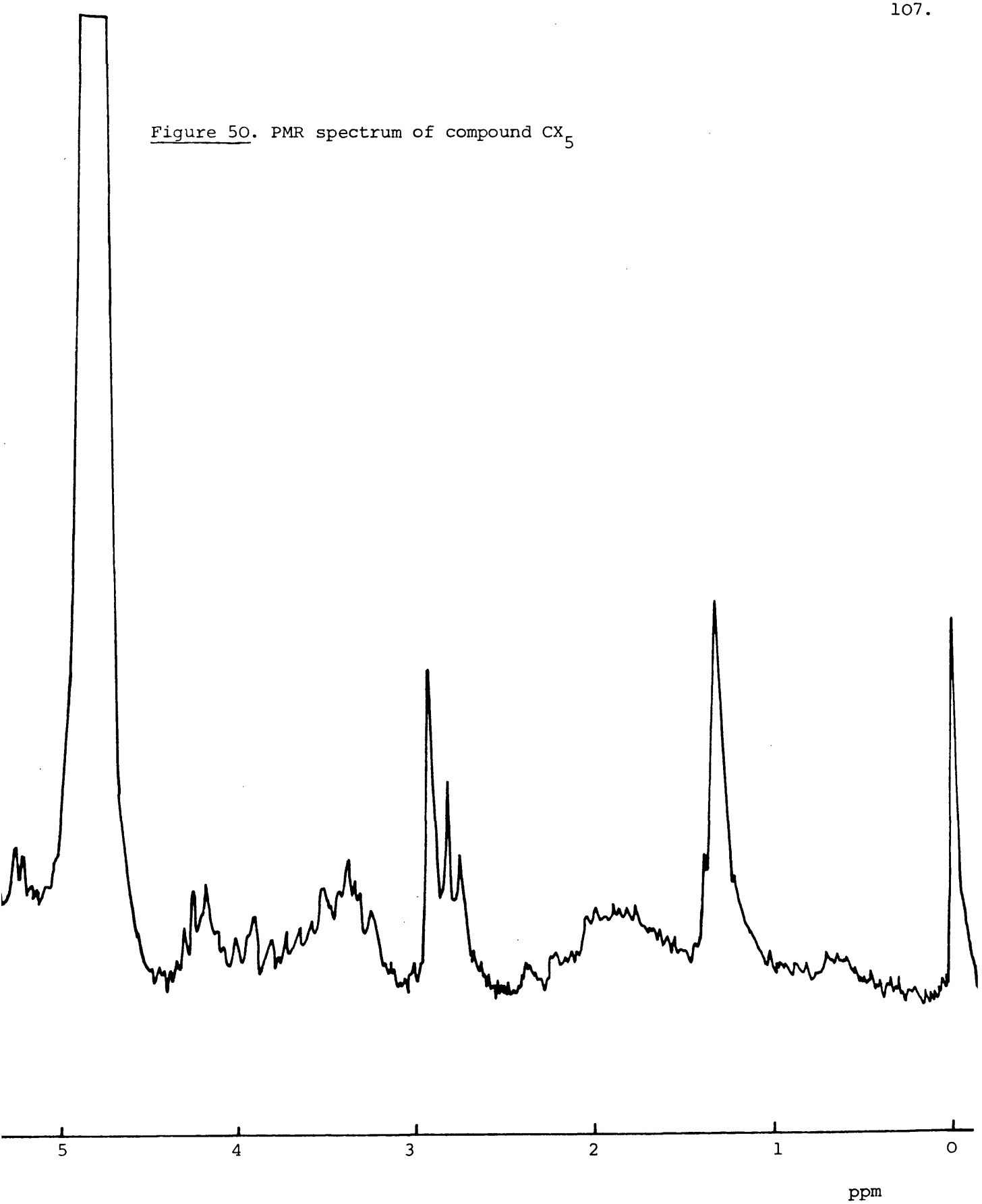



Figure 49. Field desorption mass spectrum of compound $CX_5$

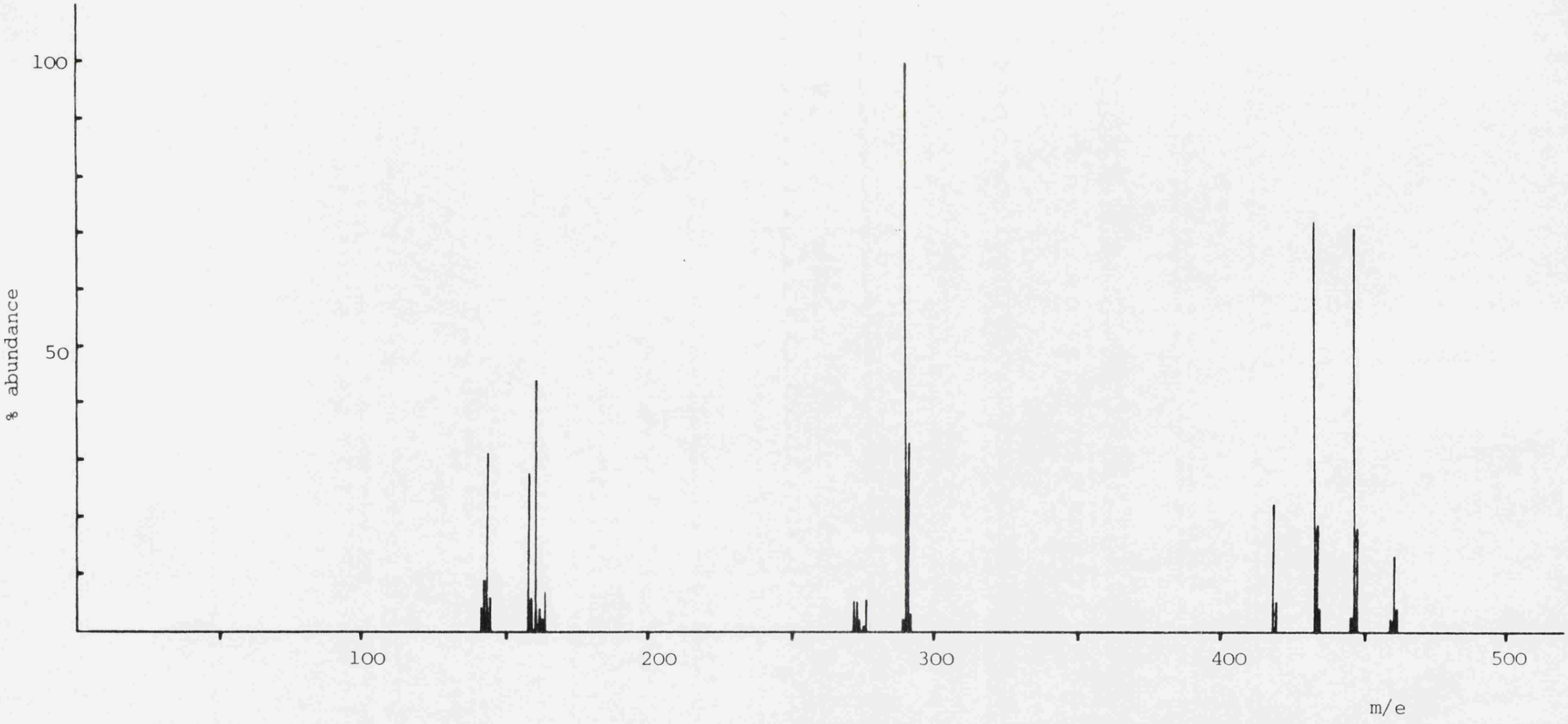
Figure 50. PMR spectrum of compound $CX_5$



8) Compound $CX_7$

Figure 51 shows the chemical ionisation mass spectrum of compound $CX_7$. It suggests a mixture of compounds differing in their degree of methylation. The lack of a peak at m/e 322, the very prominent peak at m/e 290 and the peaks at m/e 160 (garosamine moiety), m/e 157 and m/e 143 (A rings of gentamicin $C_1$ and gentamicin $C_2$ respectively) all suggest that these compounds differ from the gentamicins in having a B ring which is not 2-deoxystreptamine. Thus this mixture appears to be related to $CX_5$ and may indeed have some compounds in common.

Figure 51. Chemical ionisation mass spectrum of compound $CX_7$



# CHAPTER 3

## Analytical methods based on the amino groups of gentamicin

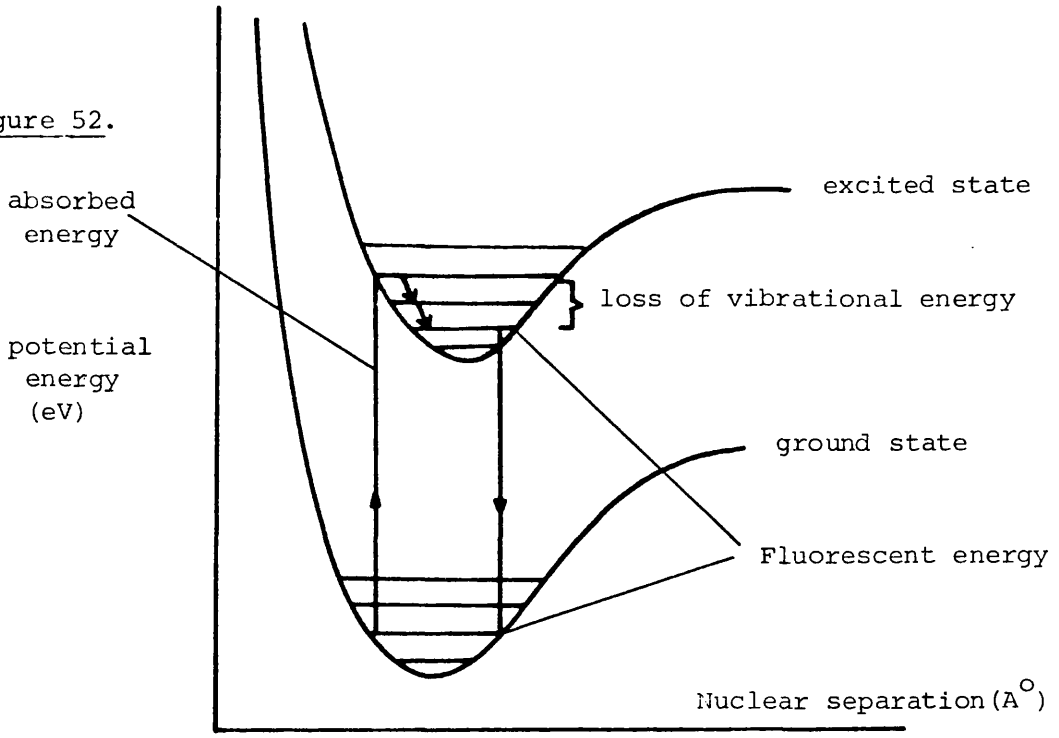
### Introduction

As an approach to the assay of gentamicin a number of analytical procedures for estimating the amino groups were examined. Experiments were carried out to determine the usefulness of:

1) Titration with acid
2) Derivatisation of the primary amino group with various fluorophores followed by fluorometric assay.

Fluoresence is a phenomenon whereby certain compounds are being irradiated with electromagnetic radiation of a specific wavelength, subsequently re-emit radiation of a longer wavelength. On absorbing radiant energy molecules are excited to a higher electronic state, and must lose this excess energy in order to return to the ground electronic state (197, 198, 199). Re-emission of energy as fluorescence occurs in compounds in which the electron system is shielded in some way from other de-activation processes such as internal conversion, collision or chemical reaction (200). Such a molecule, on excitation, may possess higher vibrational energy in the excited state than it possessed in the ground state. This vibrational energy is lost by collisional processes in the higher electronic state after which the molecule fluoresces, i.e. it drops back to the ground state with the emission of energy as radiation. The potential energy diagram (Figure 52) shows that the fluorescence energy is less than the incident energy, i.e. it is of longer wavelength.

Figure 52.




Fluorimetric methods are inherently applicable to lower concentration ranges than are spectrophotometric determinations and are thus among the most sensitive available analytical techniques. The basic difference in the sensitivity between the two methods arises from the fact that the concentration-related parameter for fluorimetry (intensity of fluorescence) can be measured independently of the power of the beam incident upon the solution (Po). In contrast, a spectrophotometric measurement is dependent upon the ratio between the intensities of incident (Po) and transmitted light (P). Thus the sensitivity of a fluorimetric method can be improved by increasing power of incident light (Po) as by further amplifying the fluorescent signal. In spectrophotometry, an increase in Po results in a proportionate change in P and thus does not alter the ratio between them. Thus no improvement of the detector signal results in no net gain with respect to absorbance value. Thus, fluorimetric methods generally have sensitivities that are two to four orders of magnitude better than the corresponding spectrophotometric procedures.

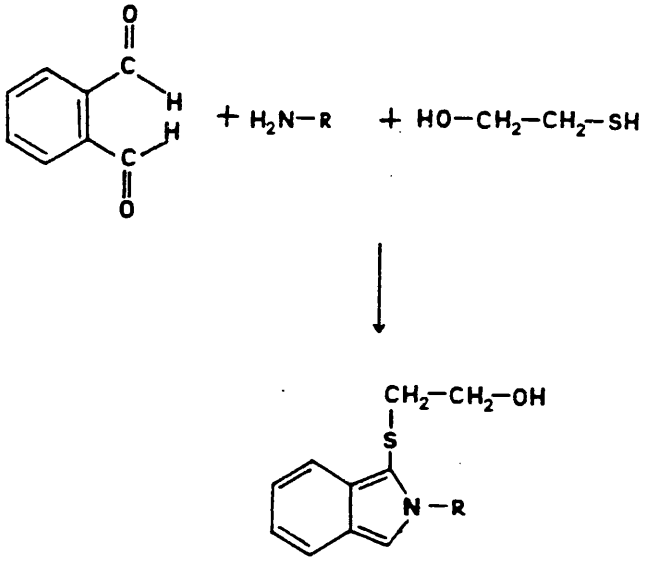
Since the excitation and emission wavelengths are characteristic of a compound, this method also provides specificity. Fluorimetry, however, is less widely applicable than absorption methods because of the relatively limited number of chemical compounds that can be made to fluoresce.

Gentamicin C itself absorbs poorly in ultra-violet or visible regions of the electromagnetic spectrum,and spectrophotometry is not applicable for the detection of therapeutic concentrations of the drug in formulations and serum. However gentamicin possesses several primary amino groups and a fluorophase can be attached readily to these groups to yield fluorescent derivatives (94, 106).

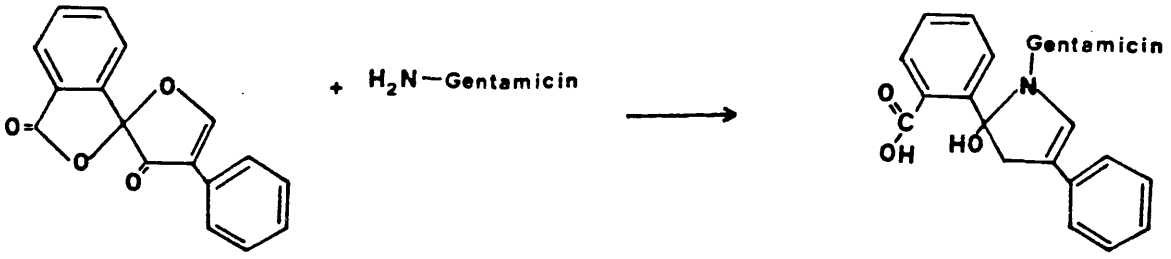
In this study ortho-phthalaldehyde, fluorescamine and acetyl-acetone with formaldehyde were used for derivatisation of gentamicin. The derivatisation reactions are shown in Figure 53.

Figure 53

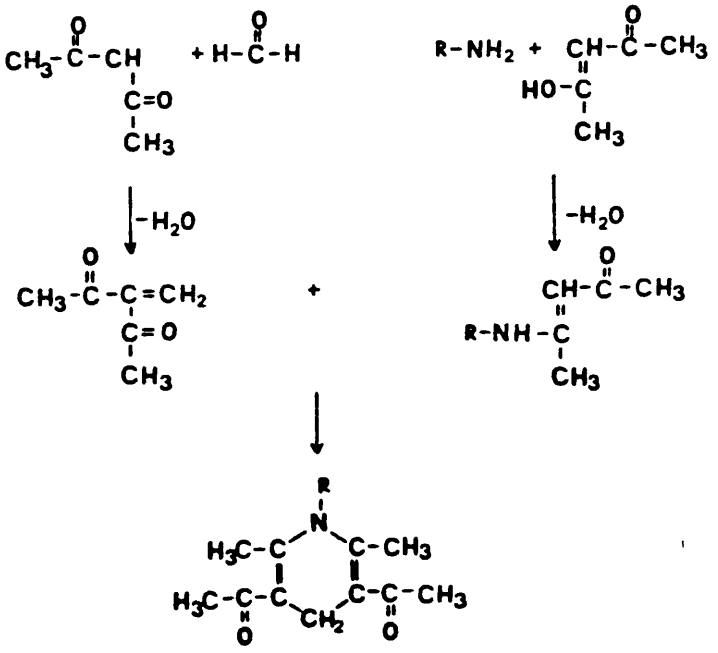
Ortho-phthalaldehyde (172-175)



Fluorescamine (201, 202)




Acetylacetone with formaldehyde (153)



## Materials and Methods

### Materials

2-Mercaptoethanol, O-phthalaldehyde, boric acid, potassium hydroxide, phosphoric acid, sodium hydroxide, hydrochloric acid, glucosamine and glycine were obtained from BDH Chemicals Limited. Methanol, acetone, formaldehyde and acetic acid were obtained from Fisons Scientific Apparatus Limited. Fluorescamine was manufactured by Sigma Chemical Company and acetyl acetone was obtained from Aldrich Chemical Company Limited.

### Instruments

For titration - pH meter (EIL Model 7050)

For fluorescence analysis - Aminco Fluoro-Colorimeter

- Model 204A Perkin Elmer Fluorescence Spectrophotometer.

### Methods

3.1 Titration of gentamicin with 0.01 N hydrochloric acid

Approximately 10 mg of gentamicin base mixture was accurately weighed and dissolved in water free of carbon dioxide. The solution was titrated with 0.01 N HCl and the pH was recorded until an obvious excess of HCl had been added.

3.2 Fluorimetric analysis of gentamicin

3.2.1 Using Fluorescamine derivatives (201)

Preparation of fluorescamine solution

Fluorescamine (10 mg) was weighed and dissolved in acetone (50 ml).

Preparation of borate buffer

0.2 M boric acid solution was adjusted to pH 8.7 with 1 N sodium hydroxide solution.

Preparation of glycine solution

In this experiment glycine was examined as a possible standard. Glycine (8 mg) was accurately weighed and dissolved in water (1000 ml) to give a concentration of 8 µg/ml. This was used to prepare a series of standard solutions of final concentrations of 0.8, 1.6, 2.4 and 3.2 µg/ml. Each solution was made up to 2.0 ml with water then 2.0 ml of borate buffer and 1.0 ml of fluorescamine solution added.

A blank was prepared by mixing 2.0 ml water, 2.0 ml of borate buffer and 1.0 ml of fluorescamine solution.

Preparation of gentamicin solution

Gentamicin C (4 mg) was accurately weighed and made up to 500 ml with water to give a concentration of 8 µg/ml. 1.0 ml of this solution was used, 1.0 ml water, 2.0 ml of borate buffer and 1.0 ml of fluorescamine solution were added.

In all cases, after addition of buffer, the tubes were rapidly shaken before addition of fluorescamine solution.

3.2.2. Using O-phthalaldehyde derivatives (115)

Preparation of O-phthalaldehyde reagent

Boric acid (1 g) was dissolved in water ( 38 ml) and adjusted to pH 10.4 with potassium hydroxide solution (450 g/litre). O-phthalaldehyde (200 mg) dissolved in methanol (2 ml)

and 2-mercaptoethanol (0.4 ml) were then added to the borate buffer solution. The O-phthalaldehyde reagent could be stored under nitrogen at $4^{o}C$ for at least five days without significant change in the fluorescence yield of gentamicin. The reagent was prepared freshly each week.

Preparation of gentamicin solutions and glucosamine solution

Gentamicin $C_1$

A 0.1 mg/ml solution in water was prepared. 0.1 ml of this solution was diluted with 8.9 ml water and 1.0 ml OPA reagent added to give a final concentration of gentamicin $C_1$ of 1 µg/ml. The reaction mixture was kept in the dark for 1 hour at $20^{o}C$.

Gentamicin $C_2$ and $C_{1a}$.

A 0.02 mg/ml stock solution of each was used. 0.5 ml of the stock solution was diluted with 8.5 ml of water and 1.0 ml OPA reagent added.

Glucosamine

Glucosamine was used as a standard in this experiment. A 0.08 mg/ml stock solution was used to prepare a series of standard solutions of final concentrations of 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6, 6.4, 7.2, 8.0, 8.8, 9.6, 10.4, 11.2 and 12.0 µg/ml. Each solution was made up to 9.0 ml with water and 1.0 ml of OPA reagent added.

A blank was prepared by using OPA (1 ml) reagent and water (9 ml).

3.2.3. Derivatisation of gentamicin with acetyl acetone and formaldehyde (153)

Preparation of Button-Robinson buffer (pH 2.6)

Solution I

Boric acid (6.183 g), acetic acid (5.73 ml) and phosphoric acid (5.6 ml) were mixed and the solution made up to 500 ml with water.

Solution II

1.0 M sodium hydroxide solution:

15 ml solution II was added to 100 ml solution I to give a final pH of 2.6.

Preparation of derivatising agent

Acetylacetone (0.8 ml) and formaldehyde (2.0 ml) were added to 10 ml of the buffer and the volume made up to 30 ml with buffer.

Preparation of derivative

A 4 ml sample of gentamicin $C_2$ (2 µg/ml) in water) and 4 ml of derivatising agent were mixed and heated for 10 minutes at 100°C.The tube was cooled and excitation and emission spectra were recorded.

Results and discussion

(1) Titration of gentamicin with 0.01 N hydrochloric acid

Figure 54 shows the titration curve of gentamicin base mixture with 0.01 N HCl. It can be seen that the end point was not sharp. A value was ascertained from a plot of $\Delta pH/\Delta V$ against value of

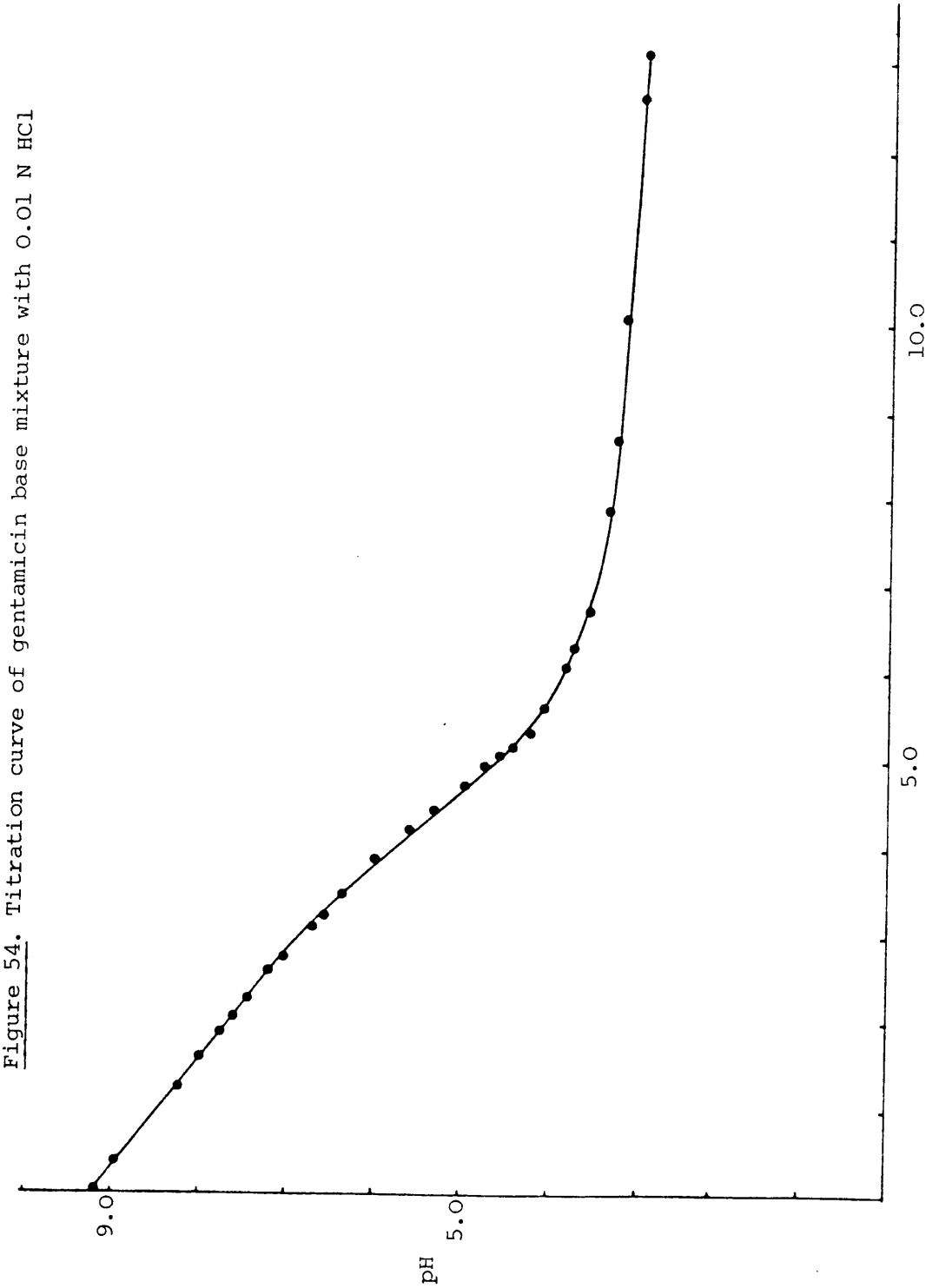



Figure 54. Titration curve of gentamicin base mixture with 0.01 N HCl

HCl added. Using this method, the amount of gentamicin in the sample was 92% of the weight taken (average of three determinations).

It was concluded that this method is not suitable for the routine analysis of gentamicin because of the difficulties in determining the end point and the rather low result for the concentration.

(2) Fluorimetric analysis of gentamicin

2.1 Fluorescamine derivative

The excitation and emission maxima of the fluorescamine derivatives of gentamicin and glycine are summarised in Table 14 It is clear that glycine is not a suitable standard since these values differ.

Table 14

| Compound | Excitation Wavelength (nm) | Emission Wavelength (nm) |
|---|---|---|
| Gentamicin | 397.5 | 475 |
| Glycine | 366 | 480 |

2.2 O-phthalaldehyde derivative

The excitation and emission spectra of gentamicin $C_2$ are shown in Figures 55 and 56. The spectra for gentamicins $C_1$ and $C_{1a}$ and the standard glucosamine were identical to these.

The calibration graph of fluorescence intensity against concentration (in moles) of glucosamine is shown in Figure 57.

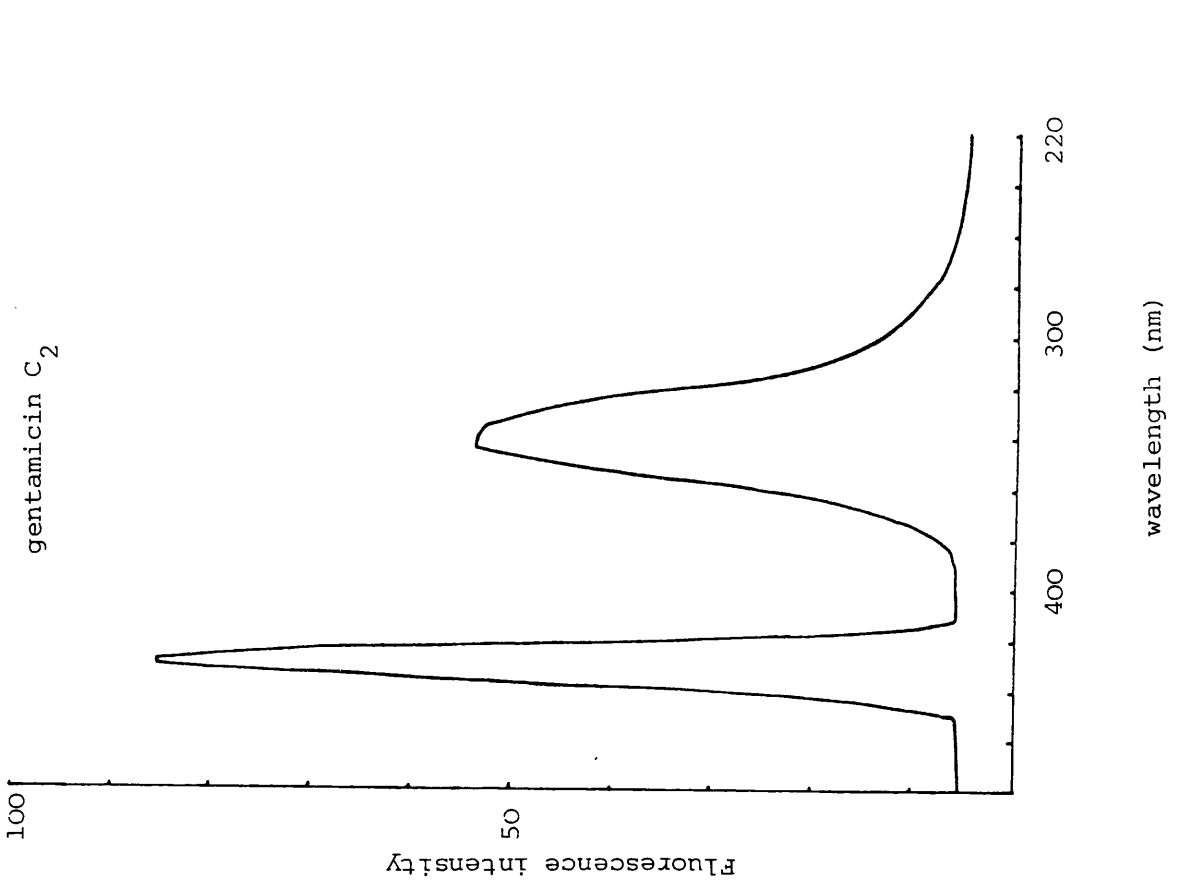



Figure 55. Excitation spectrum of derivatised

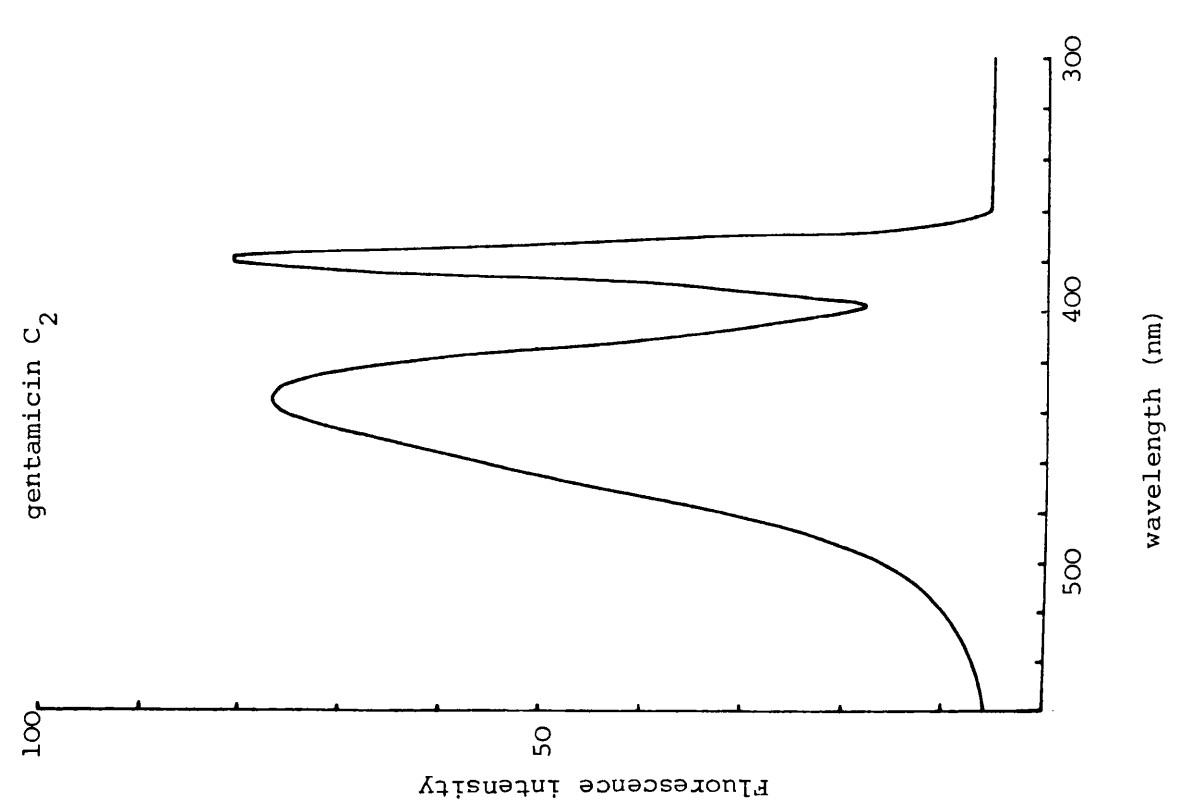
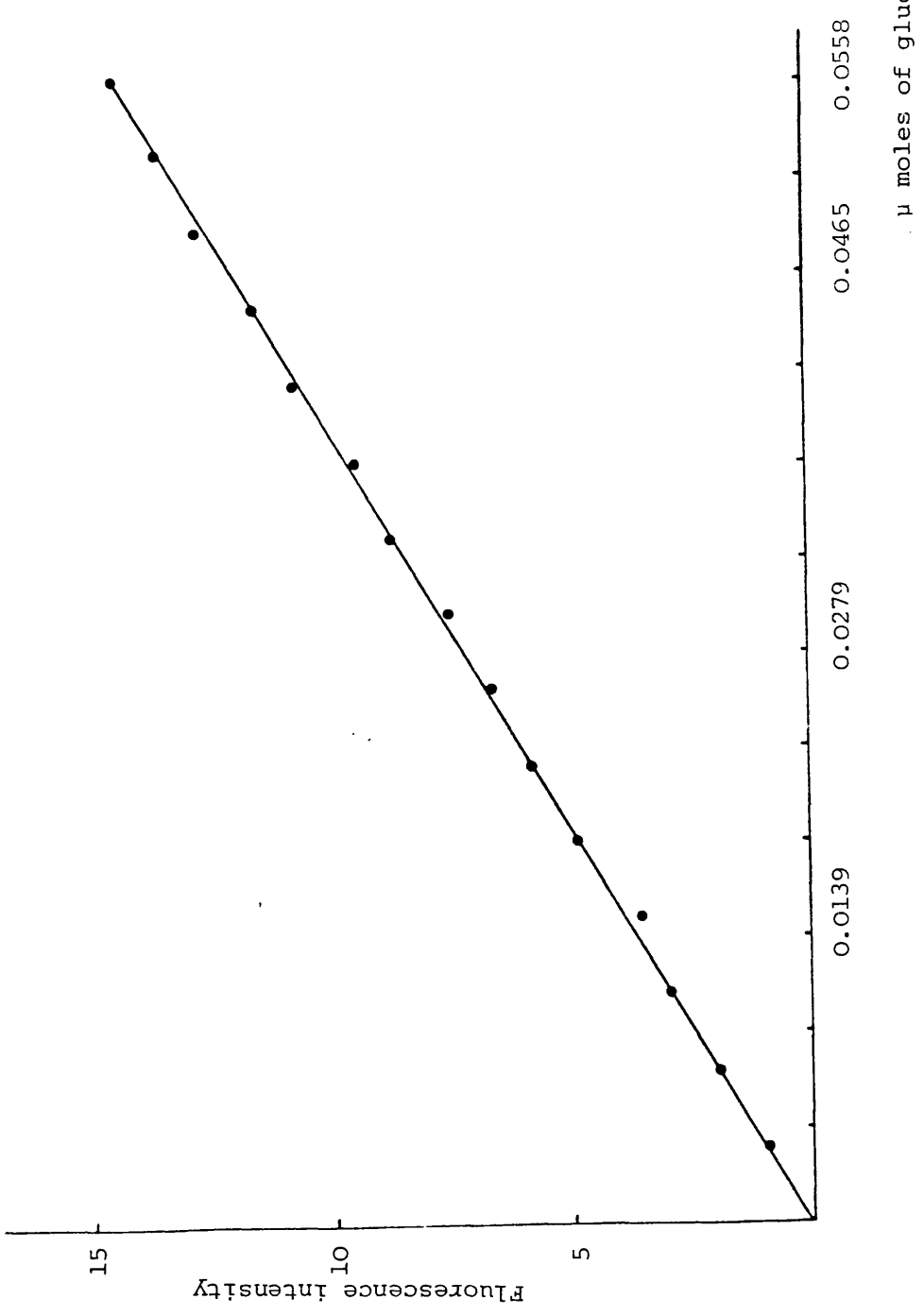



Figure 56. Emission spectrum of derivatised

Figure 57. Calibration graph of fluorescence intensity against concentration of glucosamine



The fluorescence intensities of the sample solutions were measured and the equivalent concentrations were read off from the calibration graph.

Assuming that all the primary amino groups are derivatised the equivalent weights of gentamicin $C_1$, $C_{1a}$ and $C_2$ are 159, 112.25, and 115.75 respectively.

Table 15 shows the results obtained when known samples of each gentamicin component were assayed by this method. Each value is the average of three determinations.

Table 15.

| Sample | actual concentration (µg/ml) | average fluorescence reading (arbitrary units) | calculated concentration (µg/ml) |
|---|---|---|---|
| gentamicin $C_1$ | 1.0 | 7.27 | 4.5 |
| gentamicin $C_2$ | 1.0 | 3.70 | 1.7 |
| gentamicin $C_{1a}$ | 1.0 | 3.10 | 1.3 |

It is clear that this method grossly overestimates the concentration of gentamicin.

The measured fluorescence intensity of a substance is given by the equation: (198, 200, 203)

$$F = K. \Phi . I_o . \varepsilon . c. d$$

where F = fluorescence intensity

$\Phi$ = quantum yield of fluoresence

K = constant of compounds at wavelength of measurement

$I_o$ = intensity of incident light

$\varepsilon$ = molar absorption coefficient

c = concentration

d = pathlength

In any assay in which the standard and sample are the same compound, most of these terms will be constant and fluorescence intensity is proportional to concentration within certain concentration ranges.

In this experiment in which gentamicin was compared with a model compound, glucosamine, $\Phi$, K or $\varepsilon$ may be different and thus affect the fluorescence intensity.

2.3 Lutidine derivative

The excitation and emission spectra of gentamicin $C_2$ are shown in Figures 58 and 59

Figure 58. Excitation spectrum of derivatised gentamicin $C_2$

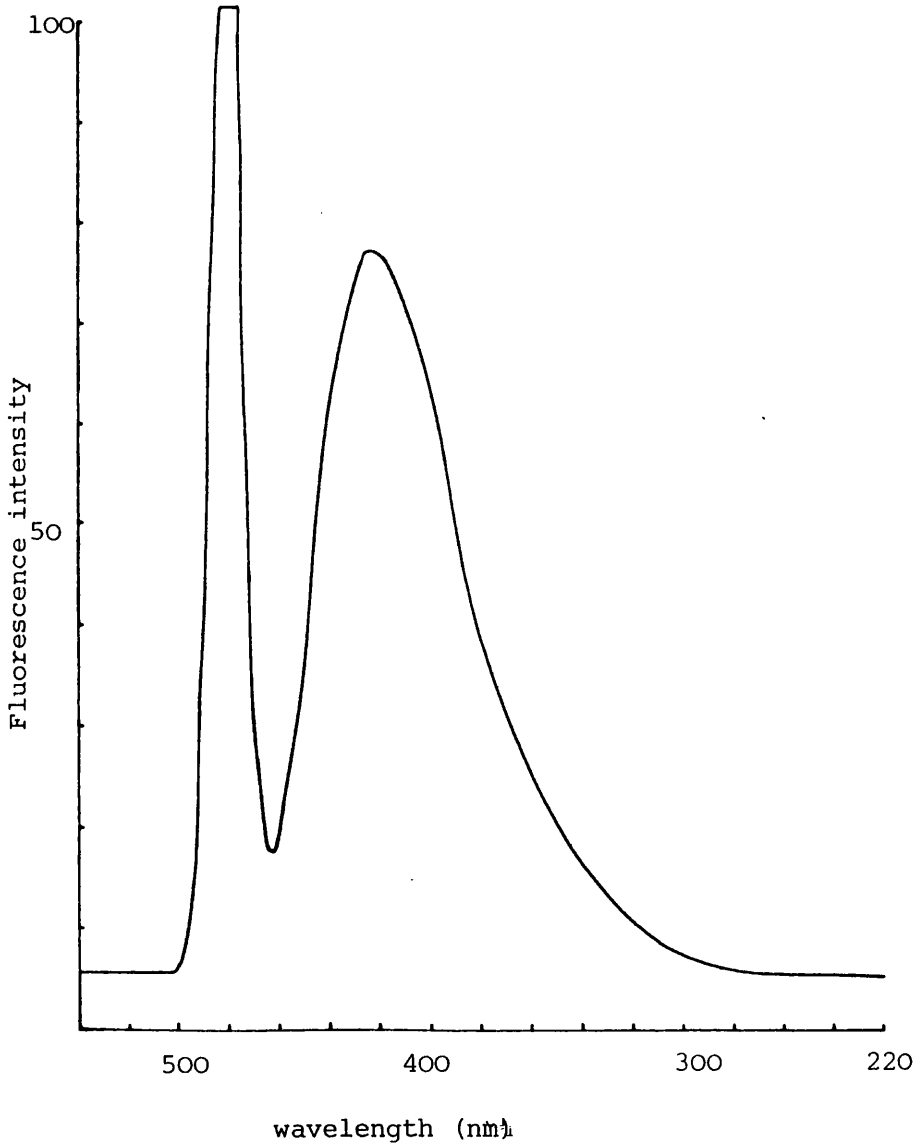
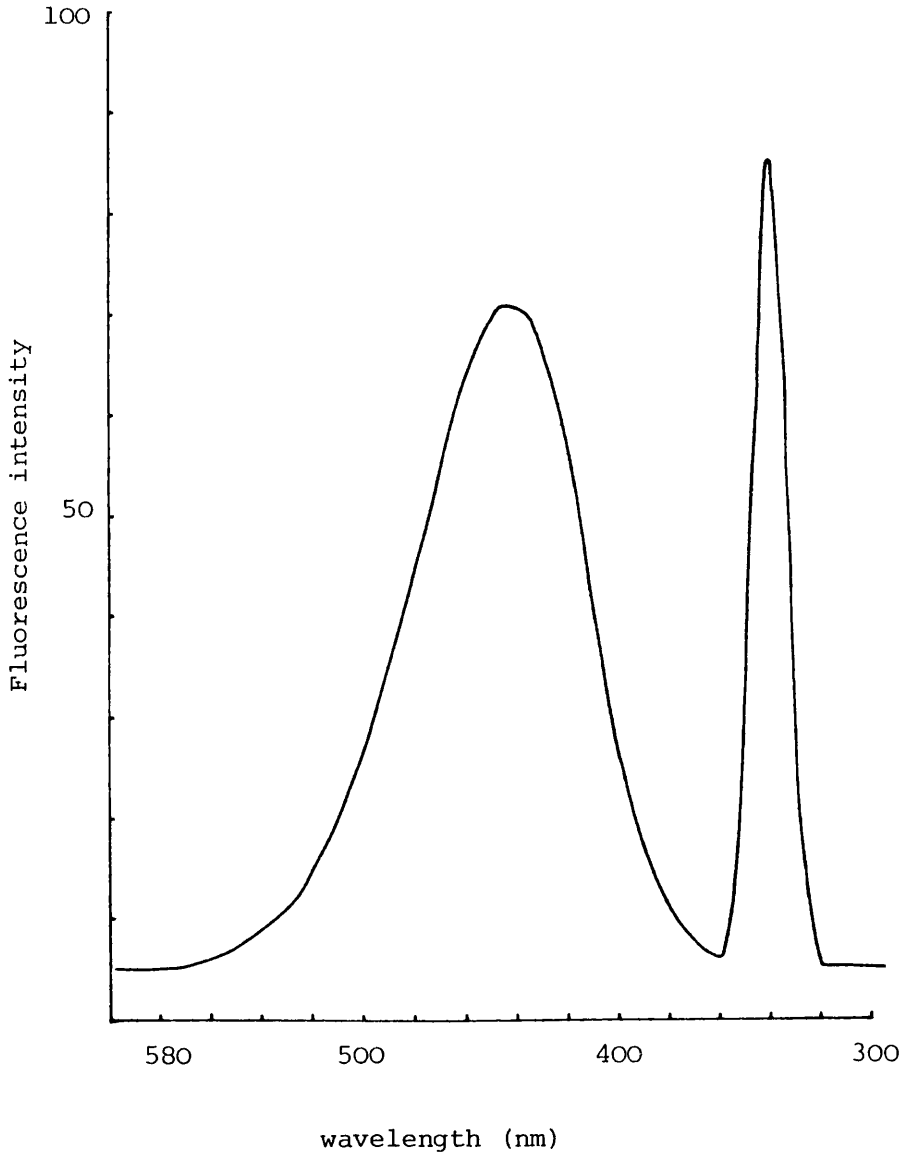



Figure 59. Emission spectrum of derivatised gentamicin $C_2$



# CHAPTER 4

## Microbiological assays and the biological activity of the gentamicin components

### Introduction

The microbiological assay used was an agar well diffusion method similar to that of the B.P. 1980 (185). In this method the substance to be assayed is allowed to diffuse through a solid culture medium inoculated with a susceptible organism. If the substance being assayed is a bactericidal agent a zone of inhibition of growth is observed. The size of the inhibition zone is a function of the concentration of the antimicrobial agent. This function can be expressed as a linear relationship between the size of the zone and the logarithm of the concentration of the substance. By measuring the diameter of the zone produced by an unknown sample and comparing it with that of a known standard preparation, the potency of the unknown sample may be calculated (204).

The advantages of diffusion type assays are: (205)

1) The method allows for more rigid control of conditions than turbidimetric methods, giving an assay of greater precision. The probable error can be readily calculated and the accuracy increased by the inclusion of sufficient replicates.
2) The testing of several samples on the same culture surface increases uniformity.
3) Samples need not be sterile. This prevents possible loss of activity from sterilisation procedures.
4) The presence of high concentrations of organic solvents can be tolerated by the use of suitable controls.

5) The method is readily automated and adapted for large scale routine analysis (207, 208).

The disadvantages are:

1) The method is relatively insensitive when compared with other methods of assay.
2) The method is limited to those substances which diffuse readily in agar. The activity of a substance is measured only to the extent of the diffusion before multiplication of the organism.
3) There may be a lack of definition of zones due to the geometry of radial diffusion.
4) The method can only be used for the comparison of samples of the same or closely related substances.
5) The method cannot be used for unknown substances until a standard has been established.

The method employed here is a cup plate method whereby the agar is allowed to harden and a slug is removed by means of a sterile cork borer. The size of the cork borer is optional. In employing this method, there are several factors which are important if accurate and reprodubible results are to be obtained. The cups must be cut vertically, and must be completely filled with the solution being assayed. The optimum thickness of the agar is 5 to 6 mm (209).

This method was used:

1) To determine the potency of a series of commercial gentamicin samples from a variety of geographical sources.

2) To determine the potency of a series of formulations containing gentamicin.

3) To compare the potencies of each of the components of commercial gentamicin isolated by column chromatography with the activity of the whole mixture.

Materials and Methods

Gentamicin sulphate was donated by Nicholas Laboratories Ltd. Sensitivity test agar (Lab m) was obtained from London Analytical and Bacteriological Media Ltd. Membrane filters were obtained from V.A. Howe and Co. Ltd.

4.1 Preparation of gentamicin solutions

Commercial samples of gentamicin sulphate were dried at 110°C for 2 hours and allowed to cool in a vacuum desiccator over silica gel. Each was dissolved in sterile distilled water to give a stock solution equivalent to 100 µg/ml of gentamicin base allowing for the sulphate content. A series of solutions were prepared to give final concentrations of 20, 10, 5, 2.5 and 1.25 µg/ml.

All the solutions were filtered through a membrane filter (pore size 0.45 µ) before transfer into the wells.

For the gentamicin-containing formulations the basic methods were modified slightly to control the effects of excipients. All formulations were obtained from Nicholas Laboratories.

4.1.1 Gentamicin injection

1 ml of injection contains:

| | |
|---|---|
| Gentamicin sulphate equivalent to gentamicin base | 40 mg |
| methylhydroxybenzoate B.P. | 1.8 mg |
| propyl hydroxybenzoate B.P. | 0.2 mg |
| sodium metabisulphite B.P. | 3.2 mg |
| disodium edetate B.P. | 0.1 mg |

5 ml of injection was diluted with sterile distilled water to a concentration equivalent to 100 µg/ml of gentamicin base. A series of solutions were prepared from this stock solution to give concentrations of 20, 10, 5, 2.5 and 1.25 µg/ml.

At the highest concentration of gentamicin, the concentrations of other ingredients were as follows.

| | |
|---|---|
| methyl hydroxybenzoate B.P. | 0.9 µg/ml |
| propyl hydroxybenzoate B.P. | 0.1 µg/ml |
| sodium metabisulphite B.P. | 1.6 µg/ml |
| disodium edetate B.P. | 0.05 µg/ml |

Another stock solution was prepared containing these concentrations of the other ingredients. This was used as a control solution.

The control wells were divided into two parts, three being filled with sterile distilled water and another three with the solution prepared above.

4.1.2 Gentamicin Paediatric

1 ml contains:

| | |
|---|---|
| gentamicin sulphate equivalent to gentamicin base | 10 mg |
| methylhydroxybenzoate B.P. | 1.3 mg |
| propylhydroxybenzoate B.P. | 0.2 mg |
| sodium metabisulphite B.P. | 3.2 mg |
| disodium edetate B.P. | 0.1 mg |

1 ml of injection was taken and diluted to 100 ml with water. This was a stock solution at a concentration of 100 µg/ml of gentamicin base. A series of solutions were prepared as in gentamicin injection. The control solution was prepared in the same way and contained:

| | |
|---|---|
| methylhydroxybenzoate B.P. | 2.6 µg/ml |
| propylhydroxybenzoate B.P. | 0.4 µg/ml |
| sodium metabisulphite B.P. | 6.4 µg/ml |
| disodium edetate B.P. | 0.2 µg/ml |

4.1.3 Gentamicin intrathecal

1 ml contains:

| | |
|---|---|
| gentamicin sulphate equivalent to gentamicin base | 1 mg |
| sodium chloride | 8.5 mg |

5 ml of the preparation was taken and diluted to 50 ml with water. A series of solutions at the same concentration as in (1) were prepared. For the control sodium chloride (8.5 mg) was accurately weighed and dissolved in sterile distilled water. The solution was made up to 50 ml.

4.1.4 Gentamicin/Hydrocortisone

Ear drops containing gentamicin sulphate equivalent to gentamicin base 0.3% w/v and hydrocortisone acetate 1%.

3.5 ml of the solution was taken and diluted to 100 ml with water. A series of solutions at the same concentration as in (1) were prepared. The solutions were passed through membrane filters, to yield clear filtrates.

4.1.5 Gentamicin Eye/Ear drops

100 ml of solution contains:

| | |
|---|---|
| gentamicin sulphate equivalent to gentamicin base | 0.3 g |
| benzalkonium chloride | 0.04 g |

3.5 ml of the preparation was taken and diluted to 100 ml with water. A series of solutions at various concentrations were prepared in the same way as (1). For the control, a 0.00028 g/100 ml solution of benzalkonium chloride was prepared.

4.1.6 Gentamicin Ointment

100 g of ointment contains gentamicin sulphate equivalent to gentamicin base 0.3 g in an anhydrous greasy basis.

The extraction method was as described in British Pharmaceutical Codex (1973) (221). Ointment (3.5 g) was accurately weighed and dissolved in chloroform (20 N). The solution was extracted with 0.2 M phosphate buffer, pH 8.0 (4 x 20 ml). The extracts were combined and the solution was made up to 100 ml with 0.2 M phosphate buffer, pH 8.0. A series of solutions at

various concentrations was prepared in the same way as (1). Chloroform vapour was removed by passing a stream of air through the solution. For the control, 0.2 M phosphate buffer pH 8.0 (20 ml) was taken and diluted to 100 ml with water.

4.2 Preparation of the plate

Agar (8 g) was weighed and placed in a bottle. Water (200 ml) was added and the mixture autoclaved at a pressure of 15 lbs/in$^2$ and 121$^{\circ}$C for 15 minutes. The agar was cooled to about 50$^{\circ}$C then 5 ml of a suspension of Bacillus pumilus NCTC 8241 (concentration of spores 10 x 10$^6$ to 100 x 10$^6$ per ml in water) were added before pouring into the plate. The plate was allowed to set and dried at 37$^{\circ}$C for 30 minutes. Wells were punched using a No. 5 cork borer (about 9 mm diameter) and a template to aid positioning of the wells.

The standard and test solutions were transferred into the wells using a 100 µl automatic pipette (Transferpette$^R$, Rudolf Brand, 698 Wertheim, West Germany) according to a Latin square randomisation (Figure 60).

| | | | | | |
|---|---|---|---|---|---|
| C | D | E | F | A | B |
| D | E | F | A | B | C |
| E | F | A | B | C | D |
| F | A | B | C | D | E |
| A | B | C | D | E | F |
| B | C | D | E | F | A |

**A, B, C, D, E and F represent concentrations of 20, 10, 5, 2.5, 1.25 µg/ml and control (sterile distilled water) respectively.**

The plates were allowed to diffuse for 2 hours at room temperature and then incubated at 37°C for 12 hours. The inhibition zone around each cup was measured by vernier caliper and a graph of log concentration against mean zone diameter was plotted. A fresh response curve was prepared each day using a sample of gentamicin B.P. (Batch Number 80601) obtained from Nicholas Laboratories as the standard.

The potencies of the unknown samples were calculated by reference to the appropriate standard response curve. Six replicates at each of five concentrations were carried out for both standards and unknowns.

Results and Discussion

Table 16 shows inhibition zone diameters for the standards and unknown samples.

It is clear that considerable day to day variation in the inhibition zone size occurs for the standard solutions, emphasising the need for the construction of fresh response curves each day. Figure 61 shows a typical calibration graph of log gentamicin concentration against zone diameter. Table 17 shows the potency of the commercial samples of gentamicin expressed as a percentage of the weight taken.

For all the samples these results were within the limit required by the B.P. 1980.

Table 18 shows the potency of the formulations as a percentage of the amount shown on the label.

Table 16 shows the relationship between zone diameter (in mm) and log concentration of gentamicin (in μg/ml).

| Sample | average inhibition zone diameter (mm) at each concentration (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 10 | 5 | 2.5 | 1.25 | Control |
| Gentamicin mixture | 24.69 | 22.61 | 20.59 | 18.47 | 15.68 | - |
| gentamicin $C_1$ | 24.77 | 22.75 | 20.70 | 18.67 | 15.51 | - |
| gentamicin $C_2$ | 25.45 | 23.58 | 21.66 | 19.57 | 16.80 | - |
| gentamicin $C_{1a}$ | 26.10 | 24.05 | 22.06 | 20.03 | 17.48 | - |
| $CX_1$ | 22.57 | 20.52 | 18.42 | 15.59 | - | - |
| $CX_2$ | 19.82 | 17.72 | 15.72 | - | - | - |
| $CX_3$ | 17.78 | 15.81 | 13.12 | 11.39 | - | - |
| $CX_4$ | 19.86 | 18.16 | 16.44 | 14.42 | 12.41 | - |
| $CX_5$ | 25.51 | 23.68 | 21.95 | 20.22 | 17.92 | - |
| $CX_6$ | 12.08 | - | - | - | - | - |
| $CX_7$ | 19.83 | 17.51 | 14.29 | 12.27 | - | - |
| $CX_8$ | 17.33 | 15.39 | 11.26 | - | - | - |
| Standard 2 | 25.28 | 23.52 | 21.70 | 19.00 | 15.70 | - |
| Pierrel 061 | 25.24 | 23.62 | 21.75 | 18.90 | 15.64 | - |
| Sample (2) | 24.83 | 23.39 | 21.49 | 18.86 | 15.70 | - |
| Chinese | 24.87 | 23.42 | 21.46 | 18.82 | 15.68 | - |
| Standard 3 | 25.11 | 23.52 | 21.33 | 18.87 | 15.93 | - |
| SZ-GMC-8-L-8 | 24.69 | 23.44 | 21.39 | 18.82 | 16.00 | - |
| Pierrel 067 | 24.81 | 23.25 | 21.22 | 18.83 | 15.69 | - |
| SZ-GMC-8-L-9 | 25.17 | 23.43 | 21.35 | 18.87 | 16.06 | - |
| Standard 4 | 25.48 | 23.36 | 21.65 | 19.09 | 15.32 | - |
| Pierrel 062 | 25.57 | 23.30 | 21.24 | 18.73 | 14.99 | - |
| Pierrel 065 | 25.07 | 23.23 | 21.50 | 19.29 | 15.49 | - |
| Pierrel 066 | 25.80 | 23.14 | 21.36 | 19.13 | 15.24 | - |
| Standard 5 | 28.95 | 26.84 | 24.58 | 22.33 | 18.48 | - |
| Pierrel 064 | 28.72 | 26.62 | 24.63 | 21.92 | 17.24 | - |
| Standard 6 | 28.18 | 26.02 | 24.15 | 21.80 | 17.90 | - |
| Hungarian | 28.01 | 25.92 | 23.85 | 21.79 | 18.22 | - |
| Standard 7 | 28.59 | 26.49 | 24.22 | 21.96 | 18.26 | - |
| Italian | 28.43 | 26.34 | 24.30 | 21.77 | 18.58 | - |
| SZ-GMC-8-L-6 | 28.42 | 26.50 | 24.28 | 21.99 | 18.50 | - |
| B/N°-GMC-8M-6080 | 28.22 | 26.18 | 24.02 | 21.91 | 17.35 | - |

Table 16 continued

| Sample | average reading (mm) concentration(µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 10 | 5 | 2.5 | 1.25 | Control |
| Standard 8 | 28.19 | 26.22 | 24.30 | 21.82 | 18.10 | - |
| SZ-GMC-8-L-7 | 28.11 | 26.17 | 24.08 | 21.89 | 18.52 | - |
| Sample (3) | 28.22 | 26.07 | 24.14 | 21.47 | 18.33 | - |
| Standard 9 | 28.47 | 26.31 | 24.29 | 21.88 | 18.06 | - |
| Sample (4) | 28.35 | 26.25 | 24.28 | 22.07 | 18.28 | - |
| Sample (1) | 28.50 | 26.40 | 24.17 | 21.59 | 18.06 | - |
| Standard 9 | 25.23 | 23.37 | 21.55 | 18.86 | 15.86 | - |
| 'Genticin injection' | 25.21 | 23.77 | 21.72 | 18.72 | 15.47 | - |
| 'Paediatric injection' | 25.26 | 23.46 | 21.58 | 18.48 | 16.05 | - |
| Standard 10 | 25.11 | 23.29 | 21.45 | 18.75 | 16.12 | - |
| 'Genticin Eye/Ear drops' | 25.17 | 23.33 | 21.58 | 18.91 | 16.13 | - |
| 'Gentisone' | 25.08 | 23.35 | 21.45 | 18.88 | 15.92 | - |
| 'Gentamicin ointment' | 24.98 | 23.18 | 21.26 | 18.64 | 15.82 | - |
| Standard 11 | 24.76 | 22.66 | 20.55 | 18.56 | 15.76 | - |
| 'Genticin intra-thecal' | 25.86 | 24.32 | 23.19 | 22.07 | 19.68 | 13.53 |

Figure 61. Calibration graph

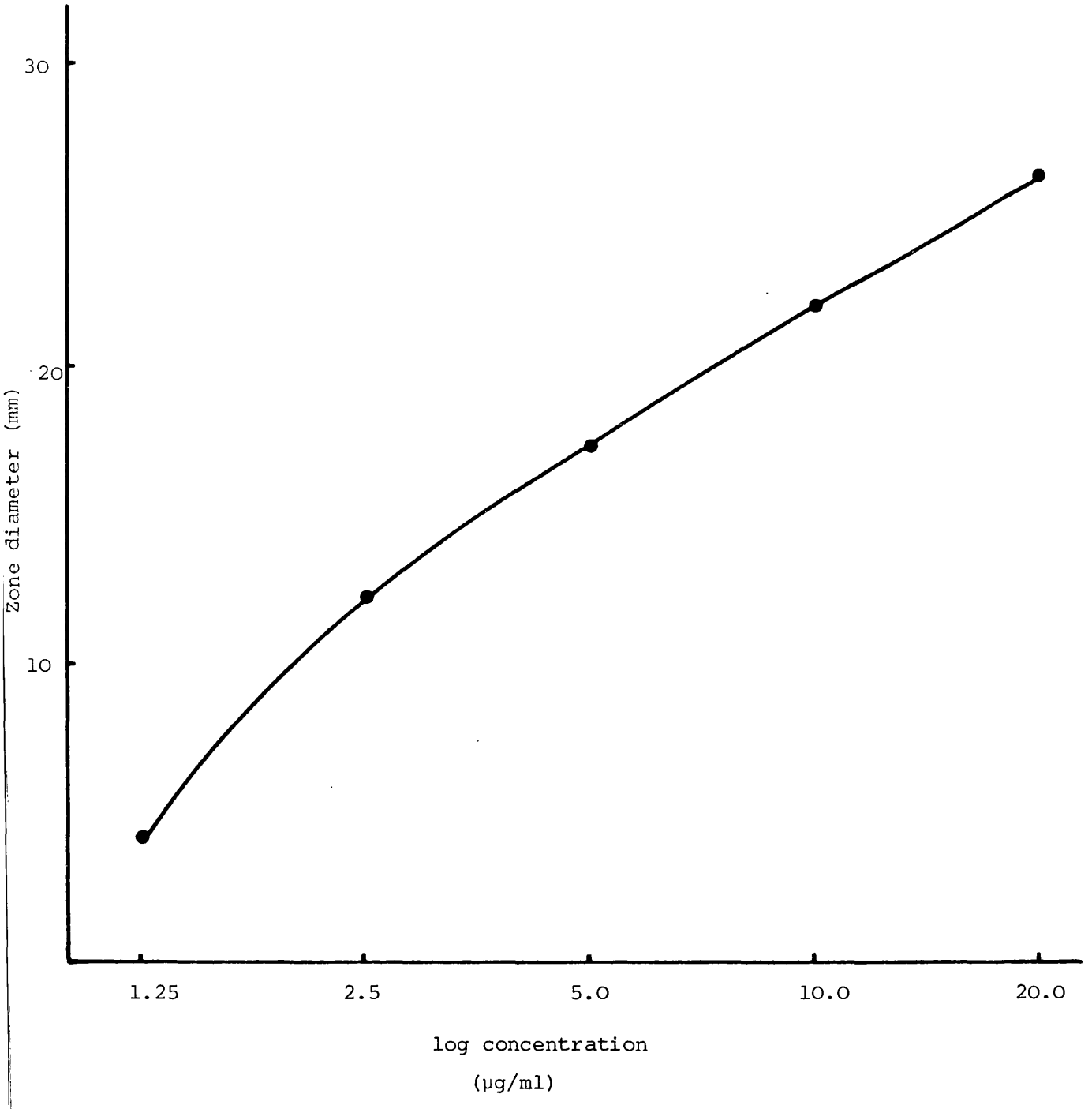


Table 17

| Sample | percentage potency | coefficient of variation |
|---|---|---|
| Pierrel 061 | 100.5 | 3.78 |
| Pierrel 062 | 97.7 | 1.99 |
| Pierrel 064 | 93.3 | 5.35 |
| Pierrel 065 | 99.9 | 8.52 |
| Pierrel 066 | 101.8 | 6.81 |
| Pierrel 067 | 95.1 | 4.65 |
| SZ-GMC-8-L-6 | 99.7 | 4.37 |
| SZ-GMC-8-L-7 | 93.6 | 5.49 |
| SZ-GMC-8-L-8 | 97.6 | 8.23 |
| SZ-GMC-8-L-9 | 100.6 | 1.31 |
| Sample 1 | 99.0 | 4.28 |
| Sample 2 | 96.1 | 7.19 |
| Sample 3 | 96.5 | 5.13 |
| Sample 4 | 100.9 | 3.99 |
| Chinese | 95.9 | 5.69 |
| Hungarian | 97.0 | 5.41 |
| Italian | 98.1 | 4.39 |
| B/N$^{O}$-GMC-SM-6080 | 91.7 | 5.47 |

Table 18

| Sample | percentage potency | coefficient of variation |
|---|---|---|
| Gentamicin injection | 101.3 | 7.56 |
| Gentamicin paediatric | 101.3 | 6.48 |
| Gentamicin Eye/Ear drops | 104.2 | 5.47 |
| Gentamicin/Hydrocortisone | 102.3 | 4.23 |
| Gentamicin ointment | 98.9 | 3.25 |

The saline controls for 'gentamicin intrathecal' were themselves found to inhibit bacterial growth (Table 16). and a plot of inhibition zone diameter against log concentration for this material was not parallel to that of the standard (Figure 62). Thus this method is not suitable for the assay of gentamicin in this formulation.

This agar diffusion method was also used to compare the antimicrobial activities of the components of gentamicin C isolated as described in Chapter 2.

Figure 63 shows plots of inhibition zone size against log concentration for both major and minor components. They are compared with a plot for gentamicin mixture in its base form.

It can be seen that the major components have similar but slightly higher activity than the gentamicin mixture. This may reflect the fact that except for compound $CX_5$ the minor components have much lower microbiological activity and therefore the total activity of the mixture is reduced in the presence of minor components.

However, the graph also shows that some of the minor components do possess antimicrobial activity.

Some of these minor components may be structurally related to the compounds isolated from crude fermentation broths by Berdy *et al.* (166) which also showed antimicrobial activity

Figure 62.

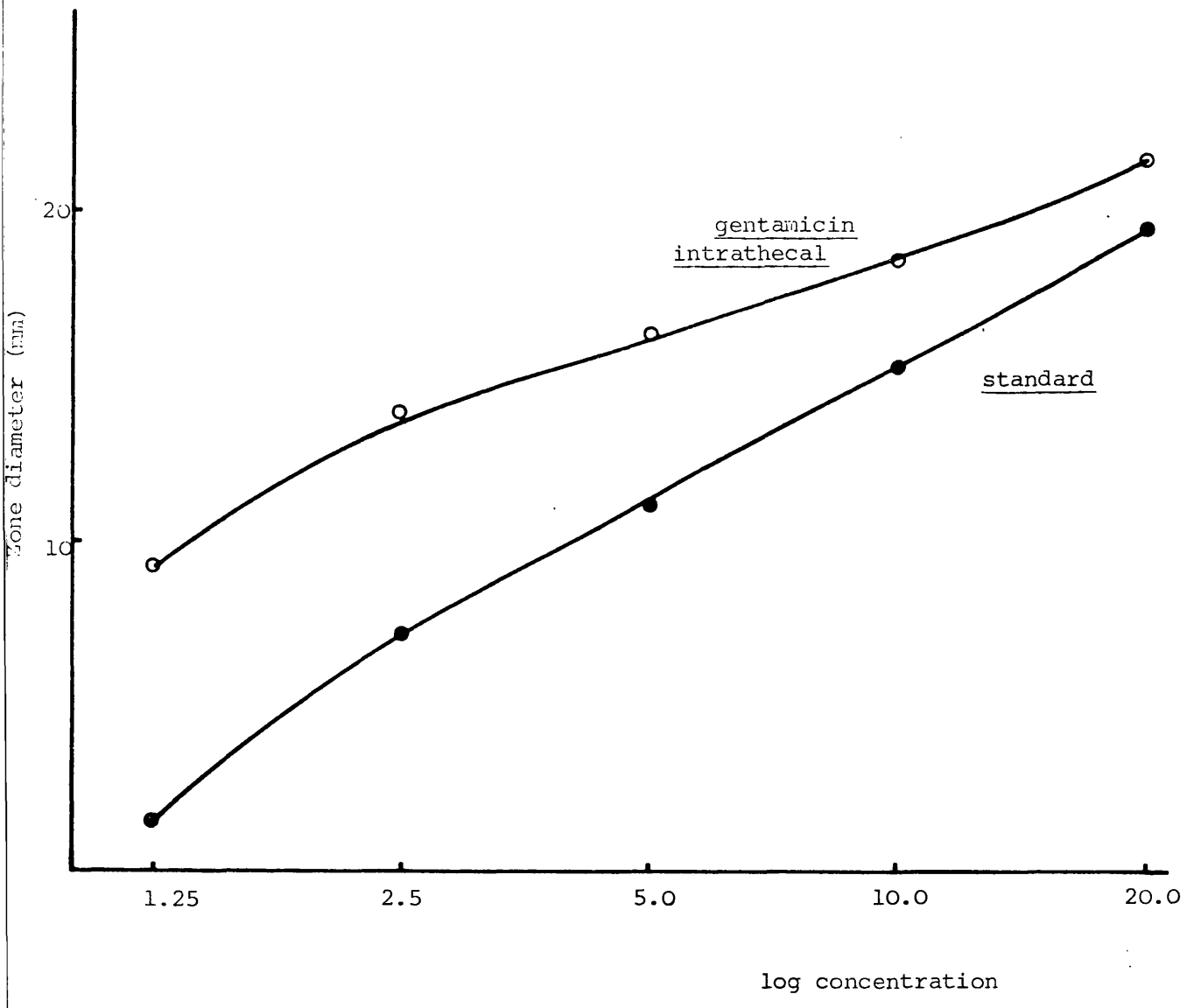
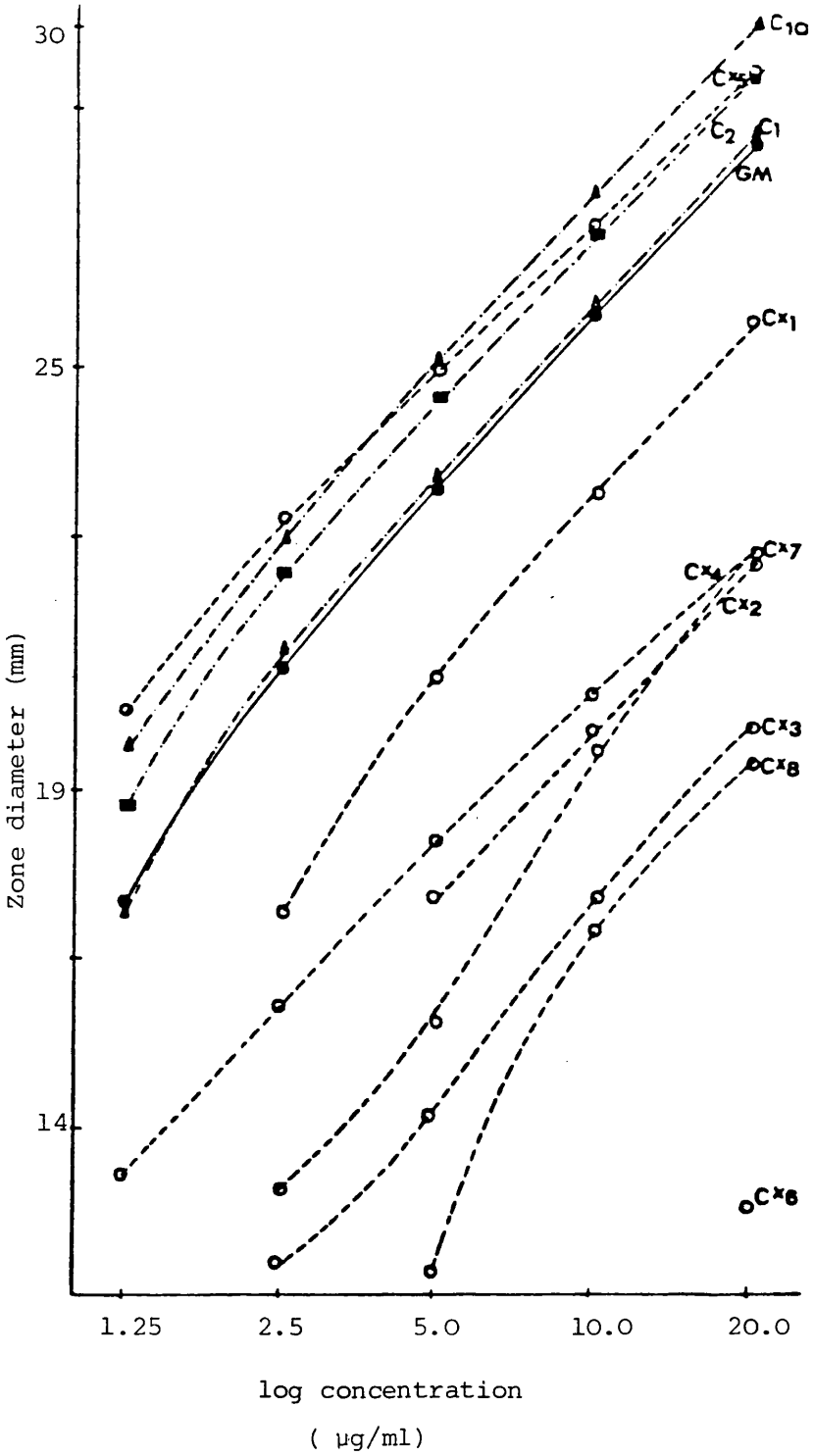

20
10
gentamicin
intrathecal
standard
1.25
2.5
5.0
10.0
20.0
log concentration

Figure 63.


30
25
19
14
Zone diameter (mm)
1.25
2.5
5.0
10.0
20.0
log concentration
( µg/ml)
C10
C5
C2
C1
GM
Cx1
Cx7
Cx4
Cx2
Cx3
Cx8
Cx6

against Bacillus subtilis. The three minor components isolated by the method of Thomas and Tappin (151) did not show antimicrobial activity against this same organism.

# CHAPTER 5

## High performance liquid chromatographic analysis of gentamicin

### Introduction

HPLC is a development of classical liquid chromatography in which the introduction of high-pressure pumps allows rapid flow rates and the use of small particle column packing materials with high efficiency. The basic equipment is shown schematically in Figure 64 and consists of a high pressure pump, an injection port a column and a detector. The detector signal is recorded on a chart strip recorder (210, 211).

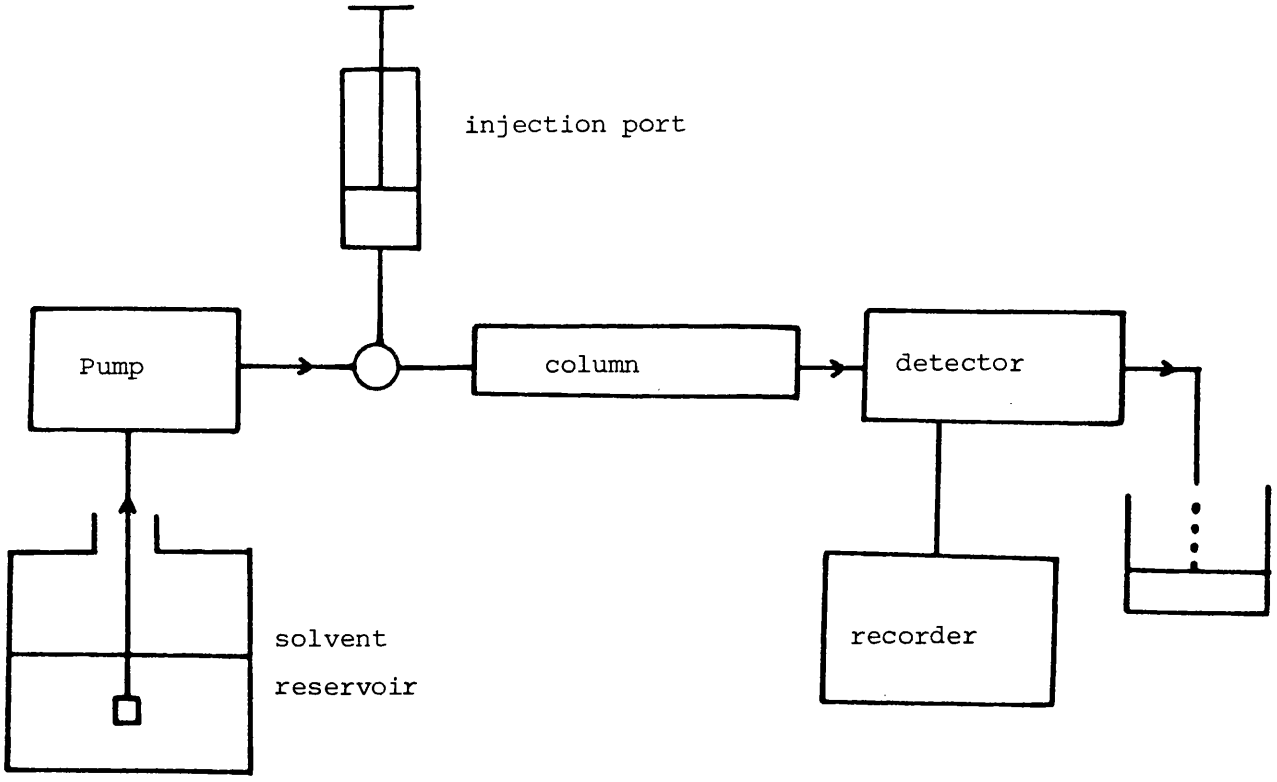



**Figure 64.** Schematic representation of equipment for high-pressure liquid chromatography

Pumping systems

There are two basic types of pump in common use: constant pressure pumps and constant volume pumps (212-215).

If a constant volume pump is used, changes in the pressure characteristics of the system, caused by settling or swelling of the packing, or viscosity changes in the mobile phase(caused either by temperature fluctuations or by composition changes) manifest themselves as changes in the pressure rather than in the flow rate. Because flow changes cause non-producible retention times, ad - versely affect resolution, and give unstable base-lines, the constant volume pump provides a more precise analysis. There are two main types of constant volume pumps: constant displacement, (syringe type) pumps and the reciprocating piston or diaphragm pumps. The former are pulse free, whereas the latter type requires pulsation dampening for detector stability.

Constant pressure pumps, consisting of some form of pneumatic device for the direct pressurization of the mobile phase with an inert gas, give a reliable pulse-free flow and have the advantage of low cost and simplicity. They are however not as accurate as constant volume pumps but can be used where flow accuracy and reproducibility are less critical.

Columns

Columns for HPLC vary depending primarily on the type of separation desired and the physical characteristics of the packing materials. Analytical columns have internal diameters of 2, 4 or

6 mm and are precision base stainless steel. The column shape is usually straight; however, with slurry packing techniques, bent or coiled columns have been shown to be equally efficient (216).

Column packings for HPLC consist of either porous layer beads (30 - 40 μm) or microparticulate packings (5 - 10 μm). Because of their higher efficiency, sample capacity, and speed of analysis, microparticulate packings are now displacing the porous layer beads for general use.

Several adsorbents have been used as packing materials: silica, alumina, graphitized and non graphitized carbon blacks, carbon-coated silica gels, and styrene-divinyl benzene copolymers (217). Alumina has some selectivity advantages in the analysis of hydrocarbons and halogen-containing compounds and is useful for highly basic compounds (218). At higher pH values alumina is more stable than silica (219).

It is possible to attach either non polar or polar groups to the hydroxyl groups on the silica surface. Thus bonded phases can be used in "normal", reversed-phase, or ion-exchange chromatography. The polarity of the bonded phase packings can vary from hydrophobic octadecylsilane to the ionic phases used in ion-exchange chromatography. Among the non polar bonded phases used in reversed-phase liquid chromatography, there are essentially four different types of bonds between the silica surface and the organic moiety attached to it (217):

(1) $-\overset{\diagdown}{\underset{\diagup}{Si}} - OR$ Ester type

(2) $-\overset{\diagdown}{\underset{\diagup}{Si}} - NR_2$ Amino type

(3) $-\overset{\diagdown}{\underset{\diagup}{Si}} - CR_3$ Carbon type

(4) $-\overset{\diagdown}{\underset{\diagup}{Si}} - O - Si - CR_3$ Siloxane type

R is usually an ethyl, octyl, or octyldecyl (ODS) group.

The shape and size distribution of commercial packings can vary from irregular to spherical particles. Both types give similar plate counts; however, spherical particles are preferred because of their higher permeability.

Injection systems (216)

Samples are introduced by one of three methods.

(1) Syringe injection

On-column syringe injection leads to the highest separation efficiency. If specially designed syringes are used, syringe injections can be made up to pressures of at least 1500 psi; ordinary microlitre syringes as used in gas chromatography can often be used up to 1000 psi. Syringe injections can be made through a septum injector or using a septum-less system.

(2) "Stop-flow" Technique

In this technique solvent flow is momentarily stopped; column inlet pressure drops to atmospheric pressure; a sample is injected onto the top of the column; and solvent flow is resumed. Normal low pressure gas chromatography syringes are used. Sample

diffusion in the solvent is so slow that resolution is unaffected.

(3) Sample valve

Valve injections are designed to operate at pressures in excess of 5000 psi without the use of septa. The solvent flow is by-passed into the column and an external or internal loop is filled with sample, which is then introduced into the column by switching a valve. The sample loops can either be external and interchangeable to give a range of volumes (10 to 500 µl), or internal and of fixed volume. Sample valves are the most convenient and reproducible method of sample injection.

Detection

Detection (and quantitation) of solutes in the column effluent can be performed by a variety of techniques including polarography, electrolytic conductivity, dielectric constant measurement, radioactivity and mass spectrometry(212). However, the most commonly used detection systems employed for liquid chromatography are absorption of ultraviolet (UV) and visible light, refractive index determination and fluorescence.

Of these, the absorption of ultraviolet or visible light is the most widely used. A wavelength of 254 nm is commonly used since many biologically important compounds absorb strongly at this wavelength. Variable wavelength UV-visible spectrophotometers covering the UV and visible light spectrum are also available.

Where a solute has no absorption in this region of the spectrum

it is often possible to prepare suitable derivatives either before (pre-colomn) or after (post column) the compound has been chromatographed.

Measurement of refractive index is the second most popular detector system in liquid chromatography. The refractometer continuously monitors the difference in refractive index between the mobile phase and the mobile phase plus the sample as it elutes from the column (213). The major advantage of refractive index detection is versatility since they do not depend upon the presence of a chromophore. However, refractometers are generally less sensitive than UV-visible light detectors by two or more orders of magnitude (214) and are not suitable for gradient elution.

Fluorescence detectors can be employed for several classes of compounds that possess natural fluorescence. Moreover, as in spectrophotometric detection, fluorescent derivatives can be prepared to detect compounds with no natural fluorescence. In this latter case the greater sensitivity of fluorescence detection often makes this preferable to attaching a UV or visible chromophore. The energy source is UV radiation, which is passed through an excitation filter for selection of the wavelength (s) at which the sample will be excited (excitation maximum). After excitation, the fluorescing sample emits energy at a longer wavelength (emission maximum). This energy is passed through an emission filter which removes the unwanted radiation, and the remainder is detected by a photomultiplier. The sensitivity of fluorimetry for strongly fluorescing compounds may approach $10^{-9}$ g/ml (213).

Application of HPLC to antibiotics

HPLC can be effectively implemented for the quantitation of antibiotics in body fluids. Necessary procedures for the assay include quantitative extraction from biological fluid, separation and accurate detection. The advantages of HPLC in the analyses of antibiotic in body fluids are (220):

(1) The wide choice of stationary phases and solvent systems available.

(2) Separations are performed within minutes and total assay time can be kept to a minimum.

(3) The method can be applied for studies of the pharmacokinetic behaviour of antibiotics.

Gentamicin has no absorption in UV or visible light, but does contain several primary amino groups to which a fluorophore can easily be attached. This provides a sensitive means of detection and by introducing lipophilic groups also allows separation by reversed-phase chromatography (107, 180, 179, 181, 177, 172, 182, 176). Two derivatising reagents, fluorescamine and O-phthalaldehyde were examined for suitability.

As stationary phase a monomolecular layer of octadecylsilane chemically bound to small particle silica gel was used. A variety of mobile phases were tested with the fluorescamine derivative. For the OPA derivative the system of Maitra et al. (172), methanol and water containing tripotassium ethylene diamine tetraacetate was used.

## Materials and Methods

### Apparatus

Analyses were performed on a liquid chromatograph (Constametric I, Laboratory Data Control, Division of Milton Roy) equipped with a fluorescence spectrophotometer (Perkin-Elmer fluorescence spectrophotometer 204-S) detector. Separations were accomplished on a 10 cm x 4.0 mm i.d. reversed-phase column (Spherisorb S5 ODS, Phase Separations) maintained at 30 $\pm$ 0.2°C. Samples were injected by the use of a 25 µl loop (Spectroscope accessory company, Sidcup, Kent, England). The liquid chromatography detector was connected to a computing integrator for measurement of the chromatogram peak areas (Perkin-Elmer Sigma 10 Chromatography Data Station). Mobile phases were filtered using a membrane filter (pore size 0.45 µ) (Satorius Membrane Filter, GMGH, 34 Gotinggen, West Germany).

### Materials

Boric acid, sodium hydroxide, tetra butylammonium hydroxide, L-arginine monohydrochloride, trichloroacetic acid, ammonium sulphate, barium perchlorate, thorin indicator, sulphuric acid and perchloric acid were obtained from BDH Chemicals Limited. Acetone, ethanol and isopropanol were from Fisons Scientific Apparatus Limited. Fluorescamine and D-camphor-10-sulphonic acid were from Sigma Chemical Company. Tripotassium ethylene diamine tetra acetate was obtained from Fluorochem Limited, Glossop, Derbys. Gentamicin $C_1$, $C_2$ and $C_{1a}$ were prepared by column chromatography as described in Chapter 2. All the components were pure when checked by thin-layer chromatography high-pressure liquid chromatography, nuclear magnetic resonance

spectroscopy and field desorption mass spectrometry. Gentamicin $C_{2b}$ was provided by Schering Corporation Research Division. Water used was glass distilled and de-ionised.

Methods

5.1 HPLC of gentamicin using fluorescamine as derivatising agent

5.1.1 Chromatographic conditions and instrumental settings

| | |
|---|---|
| Column | Spherisorb S5 ODS, 20cmx0.4cm i.d. |
| Injection volume | 25 µl |
| Flow rate | 1.8 ml/min |
| Excitation wavelength | 397 nm |
| Emission wavelength | 475 nm |
| Sensitivity varied according to each injection | |
| PM gain | 3 |

5.1.2 Preparation of mobile phase

12 different mobile phases were examined

(1) 100% methanol

(2) 80% methanol, 20% 0.1 M borate buffer pH 8.0

(3) 70% methanol, 30% 0.1 M borate buffer pH 8.0

(4) 60% methanol, 40% 0.1 M borate buffer pH 8.0

(5) 50% methanol, 50% 0.1 M borate buffer pH 8.0

(6) 40% methanol, 60% 0.1 M borate buffer pH 8.0

(7) 60% methanol, 40% of $1 \times 10^{-4}$ M cetyl dimethyl benzyl ammonium chloride solution

(8) 55% methanol, 45% of $1 \times 10^{-4}$ M cetyl dimethyl benzyl ammonium chloride solution.

(9) 60% methanol, 40% water and tetrabutylammonium hydroxide (TBA)(20 µg/ml)

(10) 55% methanol, 45% water and TBA (20 µg/ml)

(11) 60% methanol, 40% of 0.01 M D-camphor-10-sulphonic acid adjusted to pH 4.0 with NaOH

(12) 30% acetonitrile, 70% water and TBA (15 µg/ml)

5.1.3 Preparation of fluorescamine solution

Fluorescamine (10 mg) was dissolved in acetone and the solution was made up to 50 ml with acetone.

5.1.4 Preparation of borate buffer

Boric acid (1.2366 g) was dissolved in water and the solution was made up to 100 ml with water. This solution was titrated with 1 N NaOH to a final pH of 8.7.

5.1.5 Preparation of derivatives

2 ml of a stock solution of each gentamicin C component (0.08 mg/ml) was mixed with 2 ml of borate buffer and then the fluorescamine solution was added with vigorous mixing. In most experiments 1 ml of fluorescamine was used but in one experiment concerned with the extent of reaction up to 8 ml was used.

5.2 HPLC of gentamicin using O-phthalaldehyde as derivatising agent

5.2.1 Chromatographic conditions and instrumental settings

Column: Sperisorb S5 ODS, 10 cm x 0.4 cm i.d.

The column was conditioned for 1 hour, with mobile phase.

Injection volume 25 µl or 50 µl

Flow rate 1.9 ml/min or 1.6 ml/min

| | |
|---|---|
| Excitation wavelength | 345 nm |
| Emission wavelength | 430 nm |
| Sensitivity varied according to concentration in each injection | |
| PM gain | 3 |

5.2.2 Preparation of mobile phase

The mobile phase used was a mixture of methanol/water (79/21 by volume) containing 2 g of tripotassium ethylene diamine tetraacetate per litre. The mixture was passed through a membrane filter (pore size 0.45 μ) and degassed prior to use.

5.2.3 Preparation of standard solutions

A series of standard aqueous solutions of gentamicin $C_1$, $C_2$ and $C_{1a}$ were prepared to give final concentrations of 0.012, 0.02, 0.04, 0.048, 0.06, 0.07 and 0.08 mg/ml.

An aqueous solution of L-arginine monohydrochloride (0.0375 mg/ml) was used as internal standard.

5.2.4 Preparation of samples

(1) Commercial mixture of gentamicin sulphate

Gentamicin sulphate (3.5 mg) was accurately weighed and dissolved in 25 ml of distilled water.

(2) Formulations

2.1 Gentamicin Injectable

Each 2 ml of solution contains:

Gentamicin sulphate B.P. equivalent to gentamicin base 8.0 mg

Methylhydroxybenzoate B.P. 3.6 mg

Propyl hydroxybenzoate B.P. 0.4 mg

Sodium metabisulphite B.P. 6.4 mg

Sodium edetate B.P. 0.2 mg

Manufacturers: Nicholas Laboratories Limited

Roussel Laboratories Limited

Warrick Pharmaceuticals Limited

1 ml of injection was diluted to 100 ml with water. 0.1 ml of this solution was taken for derivatisation.

2.2 Gentamicin Intrathecal

Each 1 ml of solution contains:

Gentamicin sulphate B.P. equivalent to gentamicin base 5 mg

Sodium chloride Ph.Eur. 8.5 mg

Manufacturers: Nicholas Laboratories Limited

Roussel Laboratories Limited

2 ml of the preparation was taken and diluted to 10 ml with water. 0.1 ml was taken for derivatisation.

2.3 Gentamicin Eye/Ear drops

Eye/ear drops containing gentamicin sulphate B.P. equivalent to 0.3% w/v gentamicin base

Manufacturer: Nicholas Laboratories Limited

1 ml of the preparation was taken and diluted to 10 ml with water. 0.1 ml was taken for derivatisation.

2.4 Gentamicin/Hydrocortisone acetate Ear drops

Ear drops containing gentamicin sulphate B.P. equivalent

to 0.3% w/v gentamicin base and 1.0% w/v

Hydrocortisone acetate Ph.Eur.

Manufacturer: Nicholas Laboratories Limited

1 ml of the preparation was taken and diluted to 10 ml with water. 0.1 ml was taken for derivatisation.

2.5 Gentamicin cream

Containing gentamicin sulphate B.P. equivalent to 0.3% w/w gentamicin base.

Manufacturer: Nicholas Laboratories Limited.

Gentamicin cream (0.5 g) was accurately weighed and dispersed in 100 ml water. A suspension of fine particles was formed. 0.55 ml of this suspension was taken for the derivatisation.

2.6 Gentamicin Ointment

Containing gentamicin sulphate B.P. equivalent to 0.3% w/w gentamicin base.

Manufactuer: Nicholas Laboratories Limited

Roussel Laboratories Limited

Ointment (0.5 g) was accurately weighed and dissolved as completely as possible in 20 ml of chloroform. The solution was extracted with three successive 20 ml portions of 0.2 M phosphate buffer pH 8.0. The combined extract was diluted to 100 ml with buffer (221). 0.55 ml of this solution was taken for derivatisation.

2.7 Gentamicin Paediatric Injection

Each 1 ml contains:

Gentamicin sulphate B.P. equivalent to gentamicin base 10 mg

| | |
|---|---|
| Methyl hydroxybenzoate B.P. | 1.3 mg |
| Propyl hydroxybenzoate B.P. | 0.2 mg |
| Sodium edetate B.P. | 0.1 mg |
| Sodium metabisulphite B.P. | 3.2 mg |

Manufacturers Warrick Pharmaceuticals Limited

Roussel Laboratories Limited

1 ml of the preparation was taken and diluted to 25 ml with water. 0.1 ml of this solution was taken for derivatisation.

5.2.5 Preparation of O-phthalaldehyde derivatising agent (172)

Boric acid (1 g) was dissolved in water (38 ml) and adjusted to pH 10.4 with potassium hydroxide solution (450 g/l). O-phthalaldehyde (200 mg dissolved in 2 ml of methanol) and 2-mercaptoethanol (0.4 ml) were then added to the borate solution. The reagent was stored at $4^{o}C$ and protected from light. New reagent was freshly prepared each week.

5.2.6 Effect of storage time upon the stability of OPA derivatives

0.15 ml of gentamicin $C_{1a}$ solution (0.08 mg/ml), 0.1 ml of arginine solution (0.0375 mg/ml), 0.4 ml water, 0.1 ml OPA and 5.0 ml ethanol were mixed.. The solution was filtered and stored in the dark. Injections were made at the intervals of 10 minutes up to 315 minutes.

The stability of the arginine derivative in the light was also studied by using 0.1 ml arginine alone. The derivative was prepared as described above. Injections were made at the intervals of 5 minutes up to 305 minutes.

5.2.7 Preparation of derivatives

(1) For calibration graph

0.22 ml of gentamicin $C_1$, 0.18 ml of gentamicin $C_2$, 0.15 ml of gentamicin $C_{1a}$ and 0.1 ml of L-arginine monohydrochloride solutions were used in each tube. 0.1 ml of O-phthalaldehyde reagent and 5.0 ml of ethanol were added. The solutions were filtered and kept in the dark for 1½ hours before injection.

(2) For samples

0.1 ml of L-arginine monohydrochloride solution was added to 0.55 ml of each sample (in the case of those formulations where only 0.1 ml of the preparation was used, 0.45 ml of water was added) and the mixture derivatised as described above.

(3) For plasma sample

3.1 Recovery when various precipitating agents were used

3.1.1 Control

1.0 ml of gentamicin sulphate solution (0.14 mg/ml) and 3.5 ml of water were mixed using a Vortex mixer. 1.0 ml of this solution was taken, 1.0 ml of OPA reagent and 2.0 ml of ethanol were added.

3.1.2 Using trichloroacetic acid (5% w/v) as precipitating agent

1.0 ml of gentamicin sulphate solution (0.14 mg/ml) and 0.5 ml of plasma were mixed using a Vortex mixer. 3.0 ml of trichloroacetic acid solution (5% w/v) was added and the mixture was centrifuged for 15 minutes. 1.0 ml of supernatant was taken,

1.0 ml OPA reagent and 2.0 ml of ethanol were added. The percentage recovery of gentamicin was determined using a series of stock solutions containing 1, 2, 4, 6, 8, 10, 11 and 12 µg/ml of gentamicin. 1.0 ml of each solution was taken and diluted with 2.5 ml of water. 1.0 ml of each solution was mixed with 1.0 ml OPA reagent and 1.0 ml ethanol was added.

1.0 ml of the same stock solution and 0.5 ml of plasma were mixed using a Vortex mixer. 2.0 ml of trichloroacetic acid (5% w/v) was added and the mixture was centrifuged for 15 minutes. 1.0 ml of supernatant was taken, 1.0 ml OPA and 1.0 ml ethanol were added.

3.1.3 Using ethanol as precipitating agent

1.0 ml of gentamicin sulphate solution (0.14 mg/ml) and 0.5 ml of plasma were mixed using a Vortex mixer. 3.0 ml of ethanol was added and the mixture was centrifuged for 15 minutes. 1.0 ml of supernatant was taken, 1.0 ml OPA reagent and 2.0 ml of ethanol were added.

3.1.4 Using methanol as precipitating agent

1.0 ml of gentamicin sulphate solution (0.14 mg/ml) and 0.5 ml of plasma were mixed using a Vortex mixer. 3.0 ml of methanol was added and the mixture was centrifuged for 15 minutes. 1.0 ml of supernatant was taken, 1.0 ml OPA reagent and 2.0 ml of ethanol were added.

3.1.5 Using ammonium sulphate (30% w/v) as precipitating agent

1.0 ml of gentamicin sulphate solution (0.14 mg/ml) and

0.5 ml of plasma were mixed using a Vortex mixer. 3.0 ml of ammonium sulphate was added and the mixture was centrifuged for 15 minutes. 1.0 ml of supernatant was taken, 1.0 ml OPA reagent and 2.0 ml of ethanol were added.

3.1.6 No precipitating agent

1.0 ml of gentamicin sulphate solution (0.14 mg/ml) and 0.5 ml of plasma were mixed using a Vortex mixer. 3.0 ml of OPA reagent was added and the mixture was centrifuged for 15 minutes. 1.0 ml of supernatant was taken and 3.0 ml of ethanol was added.

3.2 Assay of gentamicin in a plasma sample from a patient

A 38 years old male patient from Royal United Hospital, Bath, was given gentamicin 120 mg tid. He was also receiving benzyl penicillin, erythromycin, frusemide and heparin. 1.0 ml of plasma was taken, 0.4 ml of water and 3.0 ml of trichloroacetic acid (concentration 5% w/v) were added. The mixture was centrifuged for 15 minutes. 1.1 ml of supernatant was taken, 1.0 ml OPA and 0.9 ml ethanol were added.

5.3 Sulphate assay

Where sufficient commercial gentamicin sulphate sample was available, sulphate was assayed titrimetrically (222).

Preparation of barium perchlorate solution (0.01 M)

Barium perchlorate (3.9 g) was dissolved in 200 ml of de-ionised water. 800 ml of isopropanol was added.

Preparation of thorin indicator (0.2%)

Thorin indicator (0.2 g) was dissolved in 100 ml of water.

Standardisation of the barium perchlorate solution

Isopropanol (40 ml) was added to 0.005 M sulphuric acid (20 ml) and the pH of the solution adjusted to 2.5 - 4.0 with perchloric acid (0.1 N). Thorin indicator (1 drop) was added and the solution was titrated with barium perchlorate solution to the first permanent pink.

Titration of the samples

Sample (50 mg) was accurately weighed. De-ionised water (10 ml) and isopropanol (40 ml) were added and the pH of the solution adjusted to 2.5 - 4.0 with perchloric acid (0.1 N). Thorin indicator (1 drop) was added and the solution was titrated with barium perchlorate to the first permanent pink.

Calculation

$$Ba(ClO_4)_2.3H_2O + SO_4^{-2} \longrightarrow BaSO_4 + 2ClO_4^{-1} + 3H_2O$$

1000 ml of 1 M $Ba(ClO_4)_2.3H_2O \equiv$ 96 g of $SO_4^{-2}$

or 1 ml of 0.01 M $Ba(ClO_4)_2.3H_2O \equiv$ 0.96 mg of $SO_4^{-2}$

The percentage of sulphate in a sample can then be calculated from the following:

$$\frac{0.96 \times \text{volume of barium perchlorate} \times F \times 100}{\text{weight of sample}}$$

when F is a factor to adjust the actual concentration of barium perchlorate solution to 0.01M.

5.4 Water determination (223)

Sample (0.1 g) was accurately weighed and then dried at 105°C until constant weight was obtained.

Results and Discussion

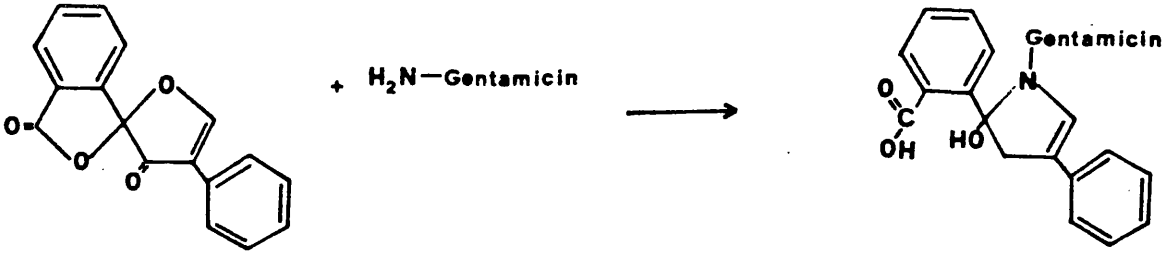
1. Using fluorescamine derivatives

When mobile phases (1) and (2) were used, each of the major components was rapidly eluted with the same retention times (30 secs with phase (1) and 1 min with phase (2)).

When mobile phase (3) was used, the retention time of each component was 1.5 minutes but gentamicin $C_{1a}$ and $C_2$ gave two additional overlapping peaks.

With mobile phase (4) and (5), all the components gave more than one peak. When mobile phase (6) was used, it was found that the retention time of each component was more than 30 minutes. There was no separation of the gentamicin components.

It has been shown that in the reaction of primary amine (gentamicin) with fluorescamine the end product has a carboxyl group as shown in the reaction




fluorescamine flurophor

Thus it was considered that a cationic ion pairing agent might achieve a separation. The reagent used was cetyldimethylbenzyl ammonium chloride; it was used to prepare mobile phase (7).

With this system the retention time of each component was 1.7 minutes and there was no separation of gentamicin components. Another cation pairing agent, tetrabutyl-ammonium hydroxide, was also examined. This was used to prepare mobile phases (8) and (9). The retention time was 1.2 minutes for mobile phase (8) and longer for mobile phase (9). Again, no separation was obtained.

Since the expected products should also contain underivatised secondary amino groups, an anion pairing agent was also examined. D-camphor-10-sulphonic acid (0.01 M) was used in mobile phase (10). All the peak heights were greatly reduced. This is probably due to the acidic pH of the mobile phase since the optimum pH for the fluorescence is between 8 - 10. If the pH of the mobile phase is increased the anion pairing agent would occur in its unsuitable ionised form. Therefore this reagent is not applicable to this case.

The acetonitrile containing systems (mobile phases 11 and 12) were also found to reduce the heights of gentamicin peaks and no separation was obtained.

The multiple peaks for each component obtained with phases 4-6 suggested that derivatisation may have been incomplete. Accordingly, an experiment was performed in which increasing amounts of fluorescamine reagent were used.

Figure 65 shows the effect of treating gentamicin $C_{1a}$ with increasing amounts of fluorescamine (analysed using phase 4). A

Figure 65. Effect of treating gentamicin $C_{1a}$ with increasing amounts of fluorescamine

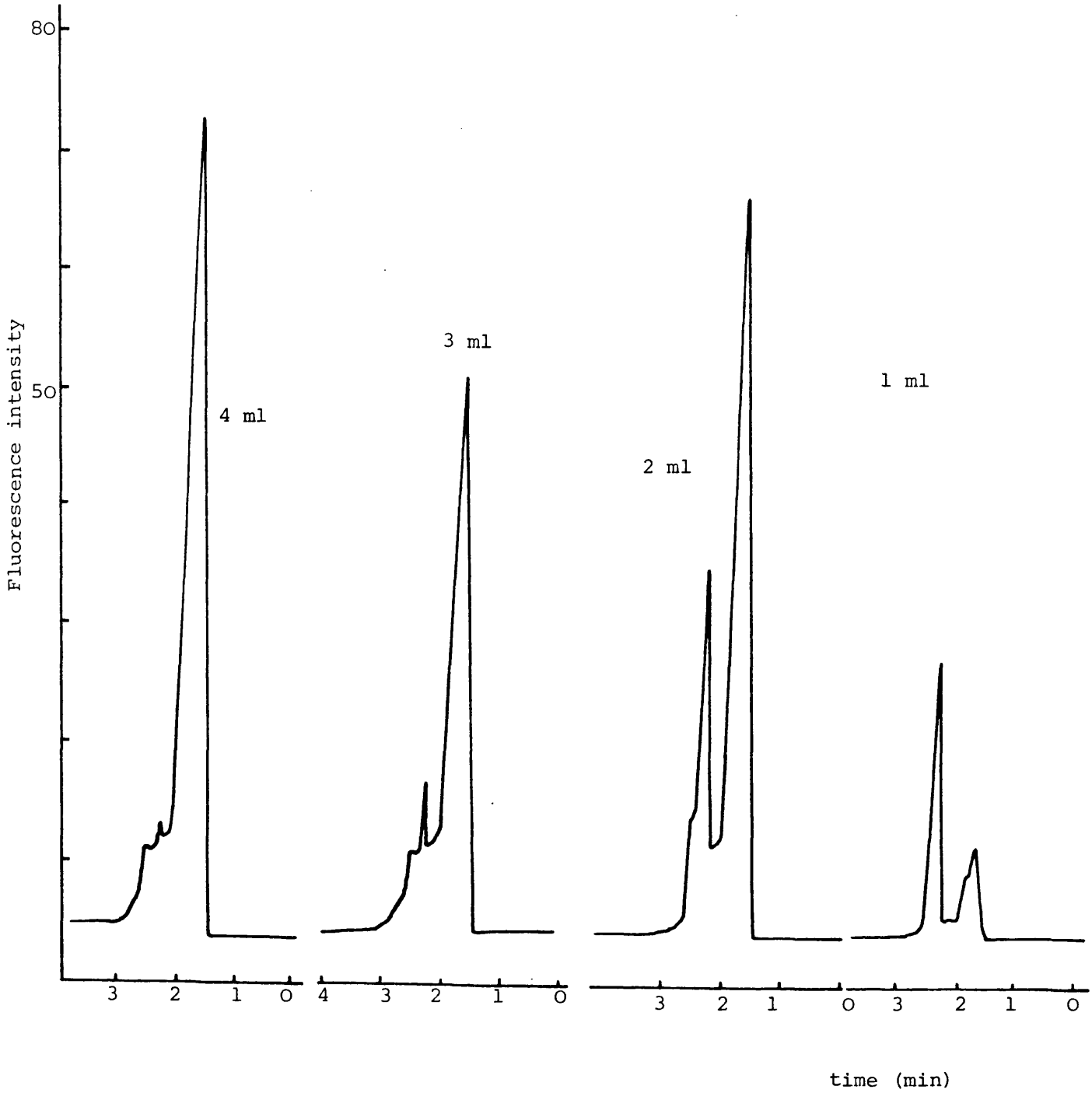
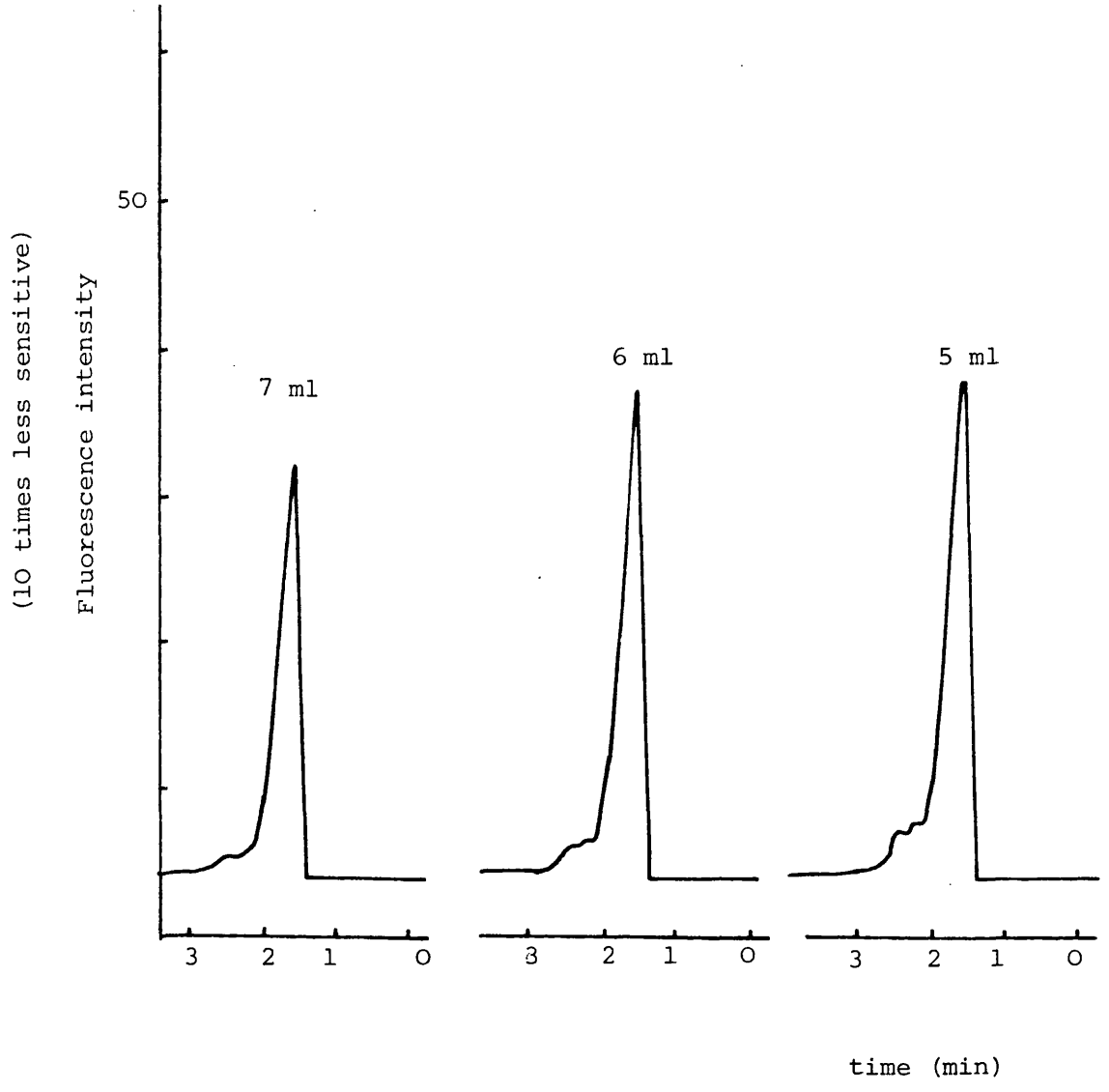


Figure 65 (continued)



similar pattern was obtained with the other major components. Thus it appears that 1 ml of fluorescamine reagent was insufficient to completely derivatise the gentamicin and that even with 6 ml of fluorescamine reagent more than one product was obtained.

It can be concluded that fluorescamine is not a suitable derivatising agent for two reasons:

(1) no separation of the compounds was obtained with any of the mobile phases used.

(2) a large excess of reagent was required for complete derivatisation.

## 2. Using O-phthalaldehyde derivatives

### (1) Development of the method

#### 1.1 Reaction conditions

Figures 66, 67, 68, 69, 70 are chromatograms of the derivatising agent alone and derivatives of arginine, gentamicin, $C_{1a}$, gentamicin $C_2$ and gentamicin $C_1$ respectively.

It was found that derivatised arginine is not stable to light, the peak area declining rapidly with time and giving rise to a complex pattern of peaks as shown in Figure 71.

Figure 72 shows that even when stored in the dark the arginine derivative undergoes some decomposition but that after about 1 hour both the peak area of the arginine derivative and the gentamicin/arginine ratio became more stable. The assay method utilises this plateau region.

The reaction worked equally well with gentamicin sulphate and the base.

Figure 66. Chromatogram of derivatising agent

Figure 67. Chromatogram of arginine derivative

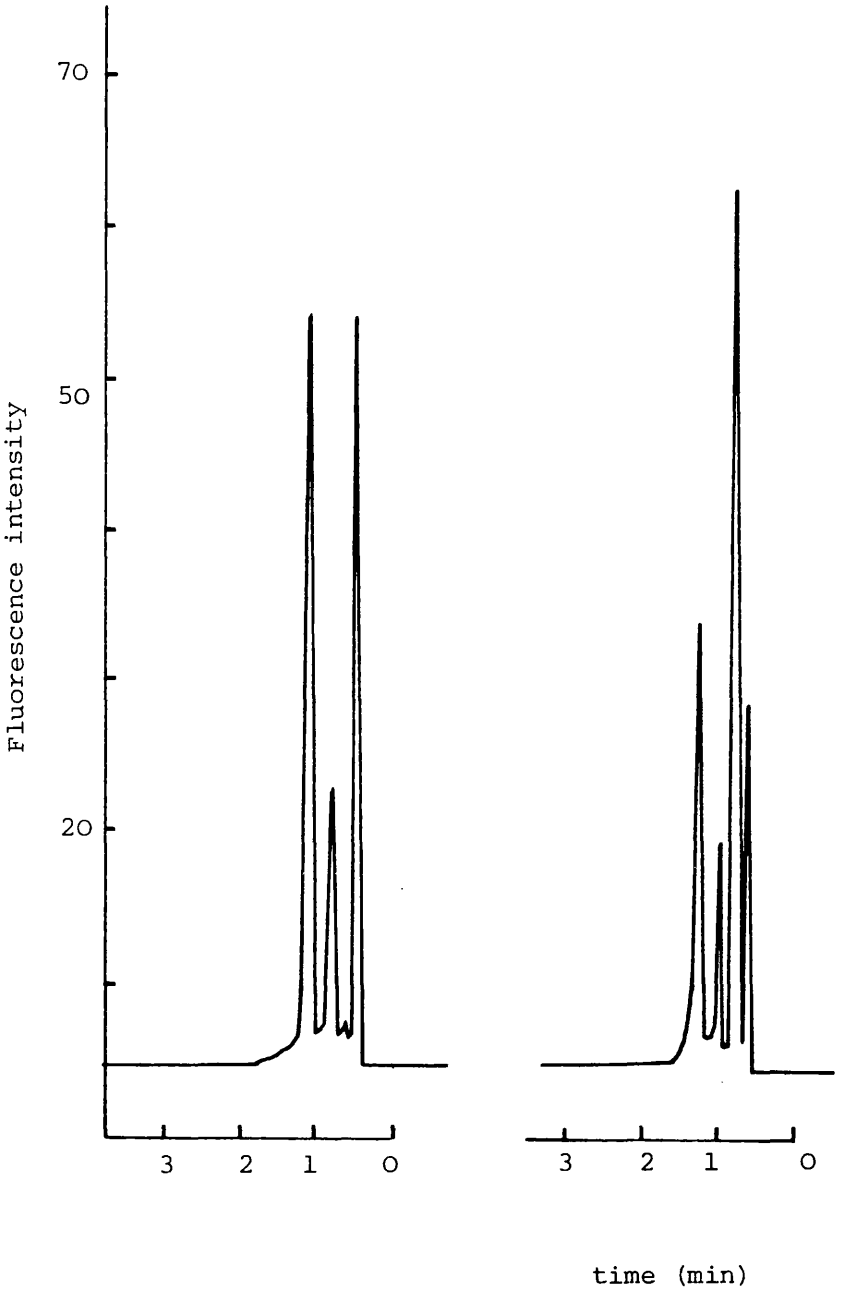


Figure 68. Chromatogram of derivatised gentamicin $C_{1a}$

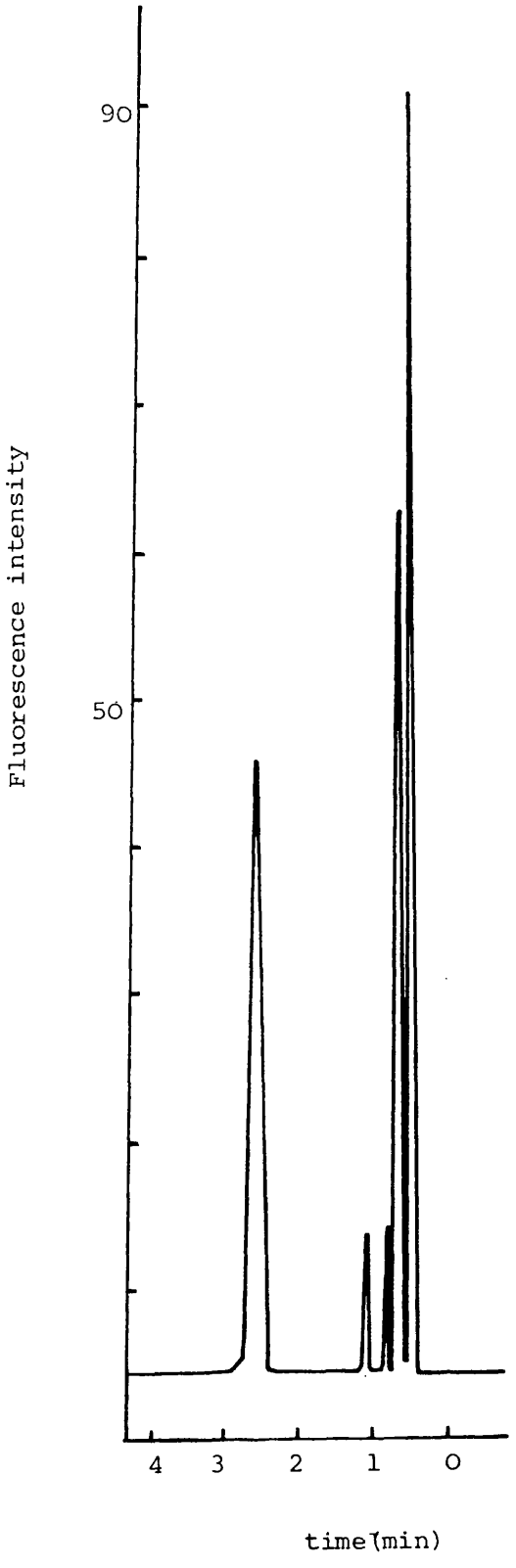


Figure 69. Chromatogram of derivatised gentamicin $C_2$

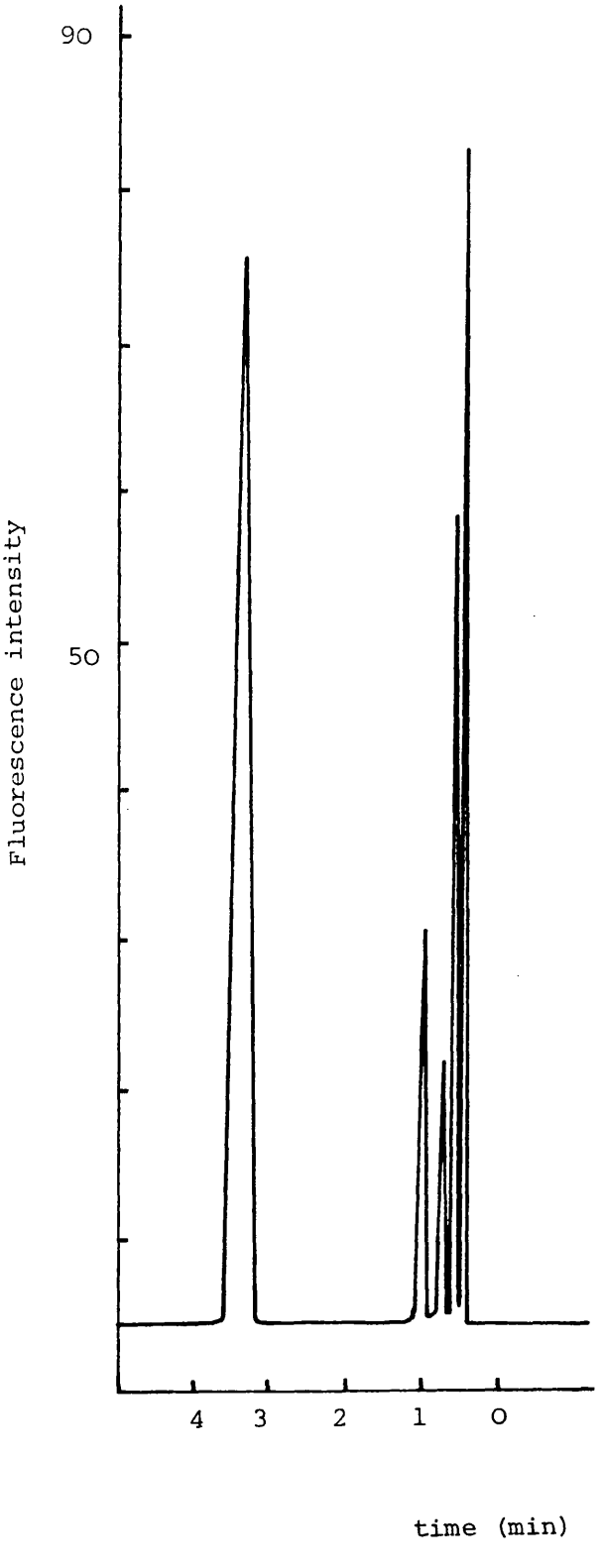


Figure 70. Chromatogram of derivatised gentamicin $C_1$

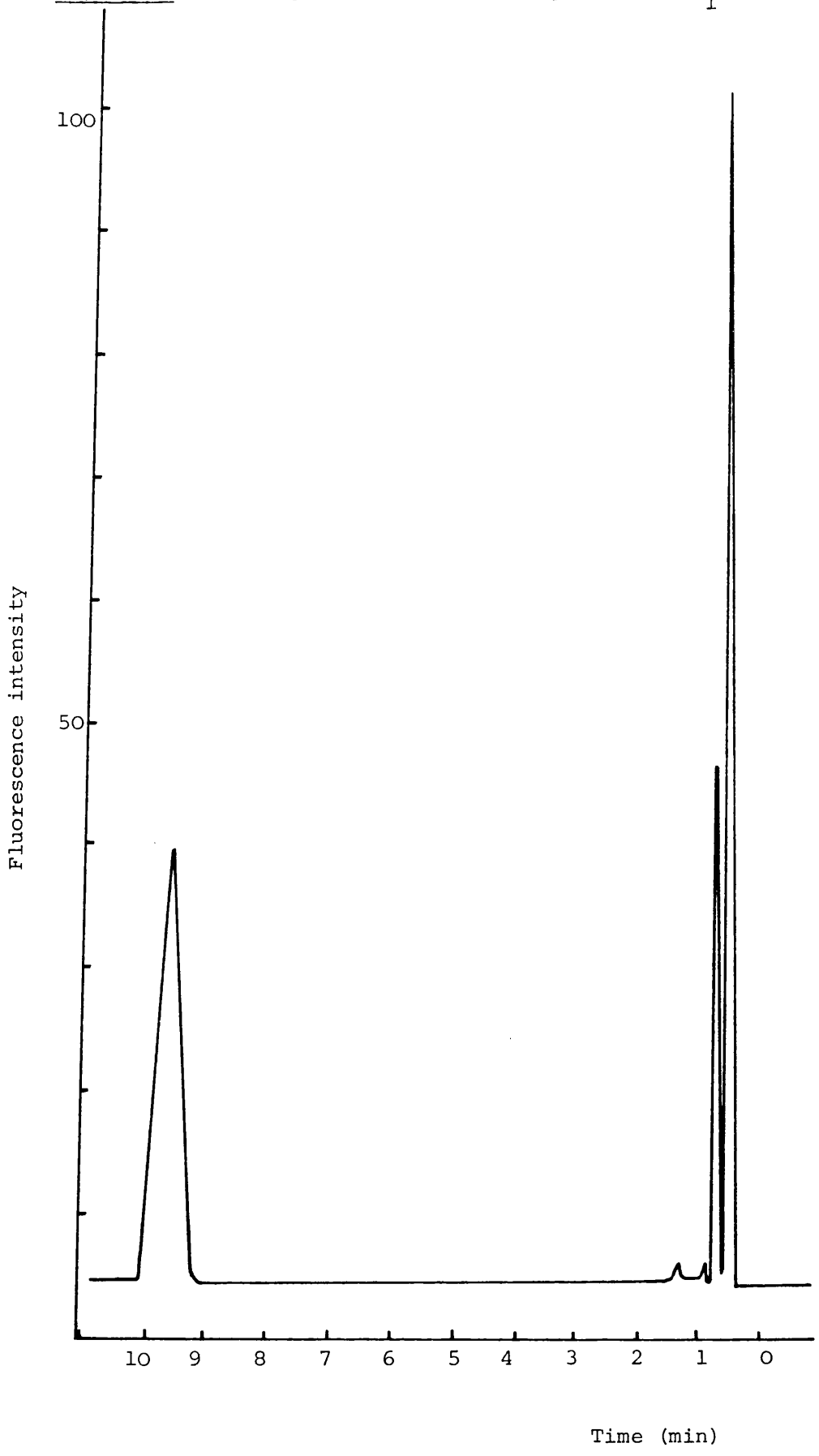


Figure 71. Effect of storage time (in the light) on derivatised arginine.

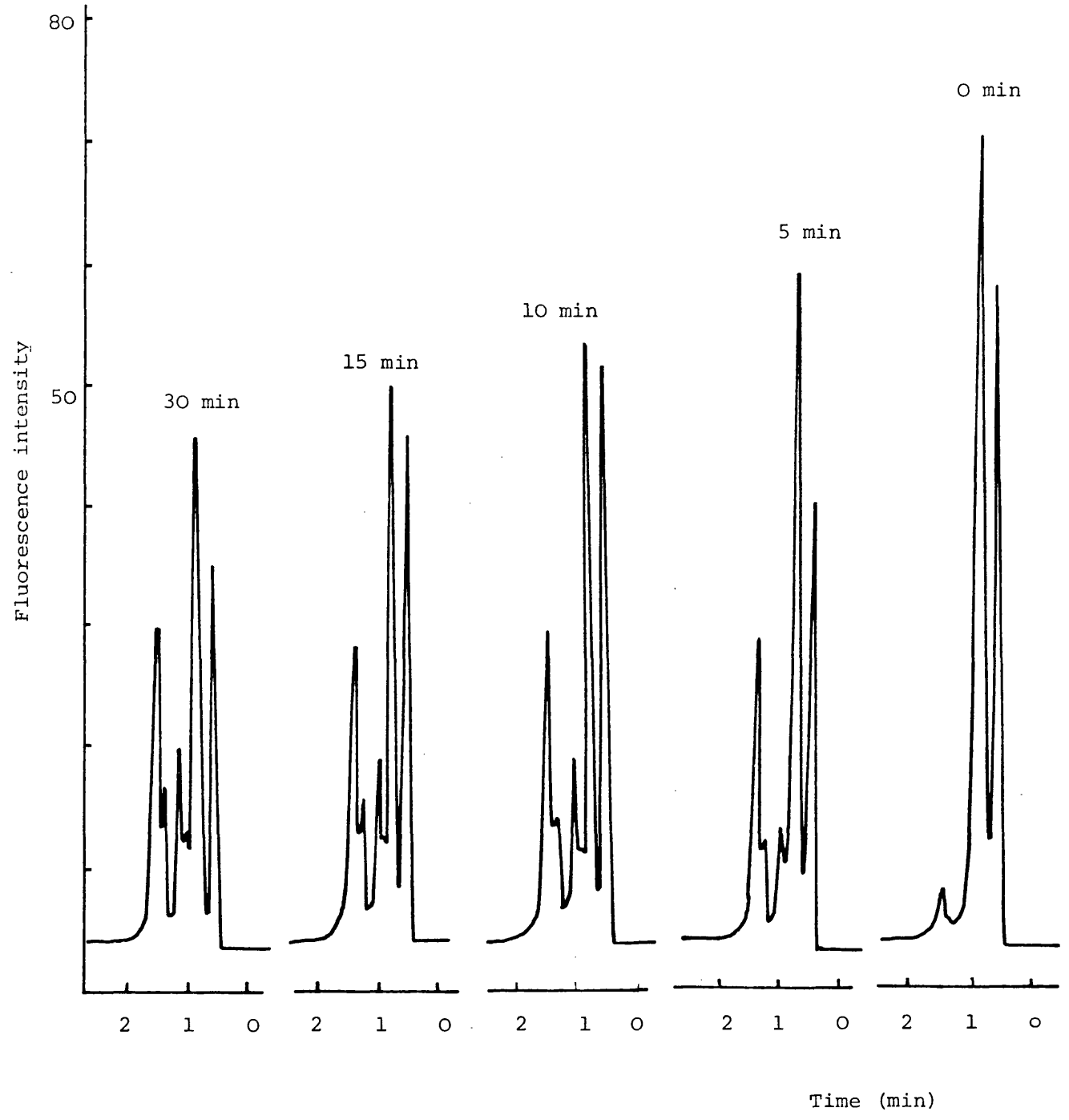


Figure 72. Effect of storage time (in the dark) on derivatised gentamicin and arginine.

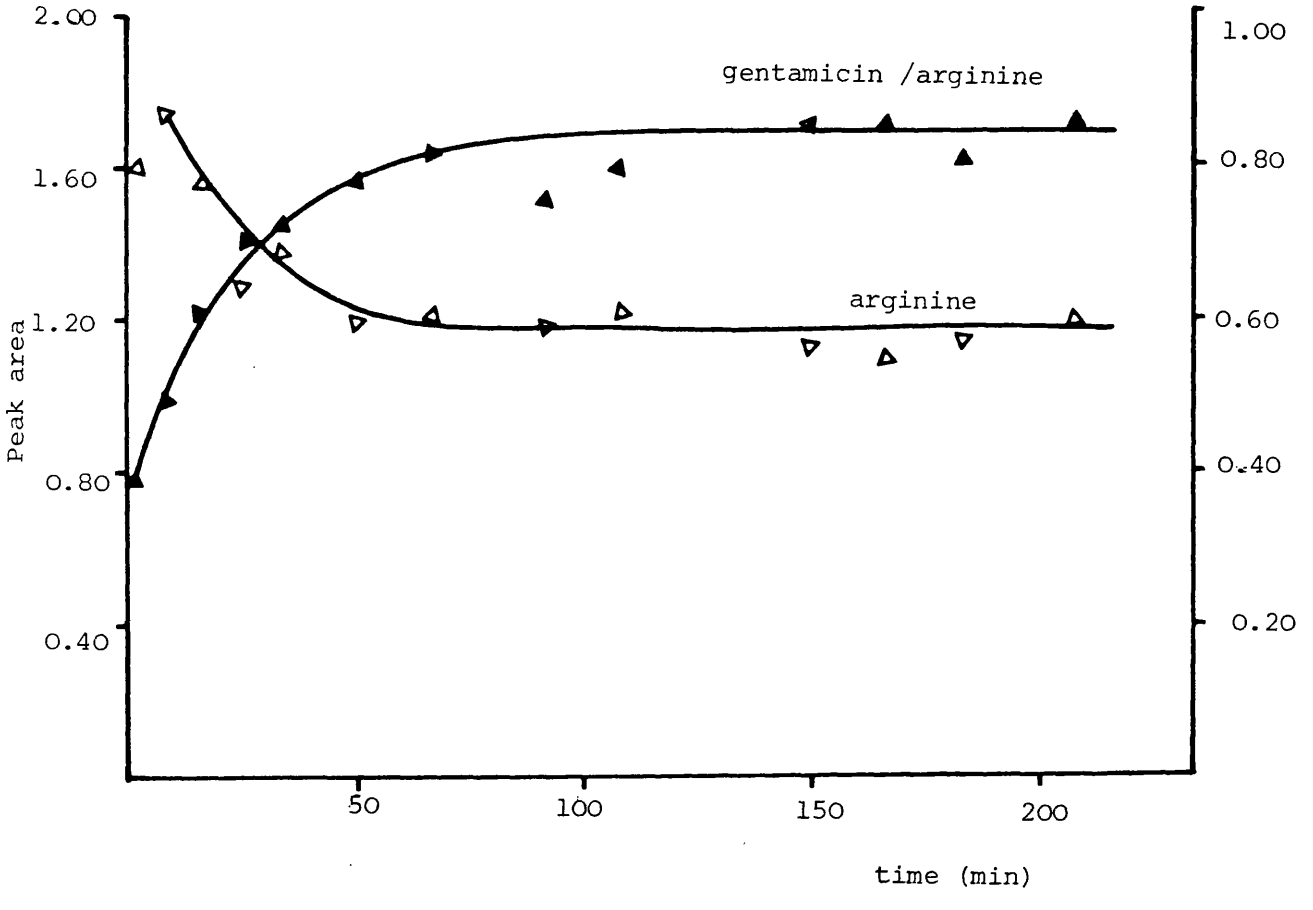


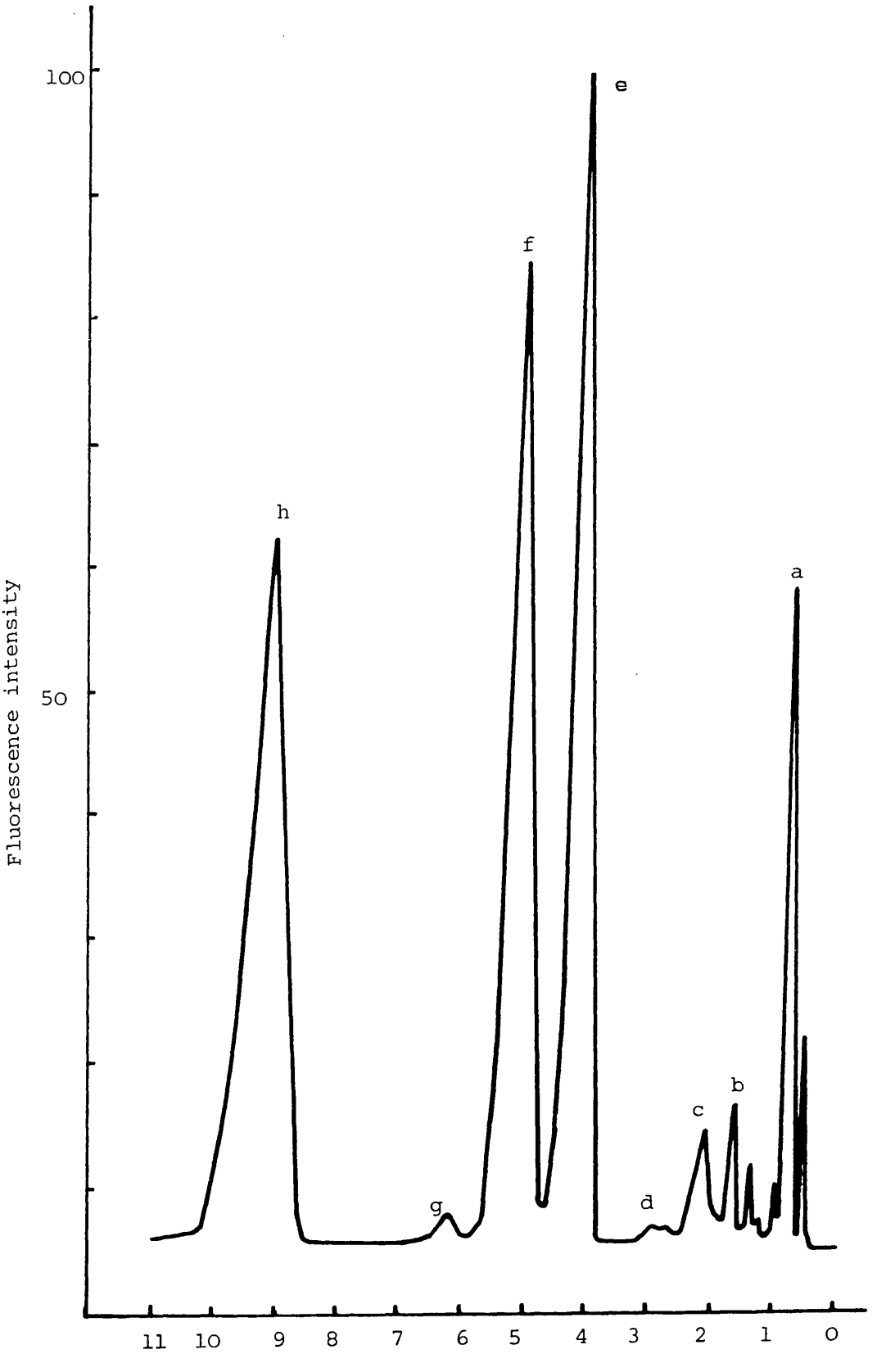
Figure 73 is a representative chromatogram obtained by this method. The elution order for the mixture was arginine, polar components, gentamicin $C_{1a}$, gentamicin $C_2$, gentamicin $C_{2b}$ and gentamicin $C_1$. It is noteworthy that this order differs from that reported by Maitra et al.(172) using a similar HPLC system. The method of Maitra et al. differs in several details from this method.

(1) Maitra et al. carried out the derivatisation reaction with the gentamicin adsorbed to silicic acid. They did not discuss the time course of the reaction. Thus it is possible that the fluorescent derivatives obtained by these workers were not identical to those used in the present study.

(2) Although both studies used a C-18 chemically bonded reverse-phase column these were obtained from different manufacturers. Several workers, in studies involving polynuclear aromatic hydrocarbons, steroids and polynuclear aromatic epoxide adducts to nucleosides, have observed differences in selectivity in C-18 chemically bonded stationary phases from different commercial sources (224-227).

(3) Maitra et al. ran their column at ambient temperature whereas in the present method the column was run at 30°C. Since increasing temperature reduces carrier viscosity and increases diffusion rate, column efficiency can be increased by increasing temperature (228). However, it was found that although the use of an elevated temperature improved resolution it did not alter the elution order.

Figure 73. Representative chromatogram of gentamicin mixture.

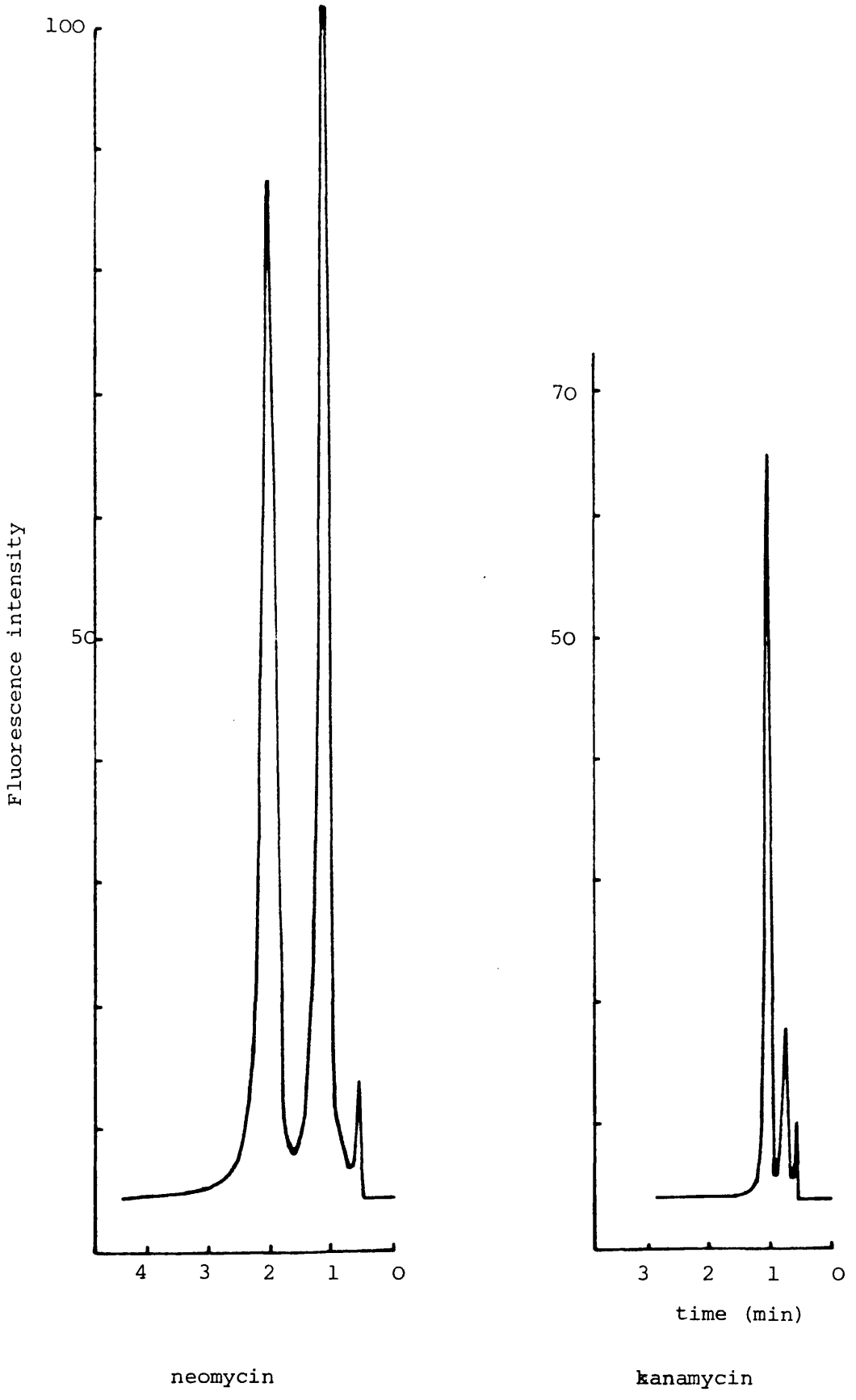



a =arginine
b,c,d = polar impurities
e = gentamicin $C_{1a}$
f = gentamicin $C_2$
g = gentamicin $C_{2b}$
h = gentamicin $C_1$

## 1.2 Interfering antibiotics

The specificity of the assay was investigated with respect to other aminoglycoside antibiotics, such as neomycin, kanamycin, and streptomycin which might be expected to form similar derivatives to gentamicin. Figure 74 shows the chromatograms of derivatised neomycin and kanamycin; no peak was observed for derivatised streptomycin. Both kanamycin and neomycin elute well before any of the major gentamicin peaks but the kanamycin peak does overlap with that of arginine.

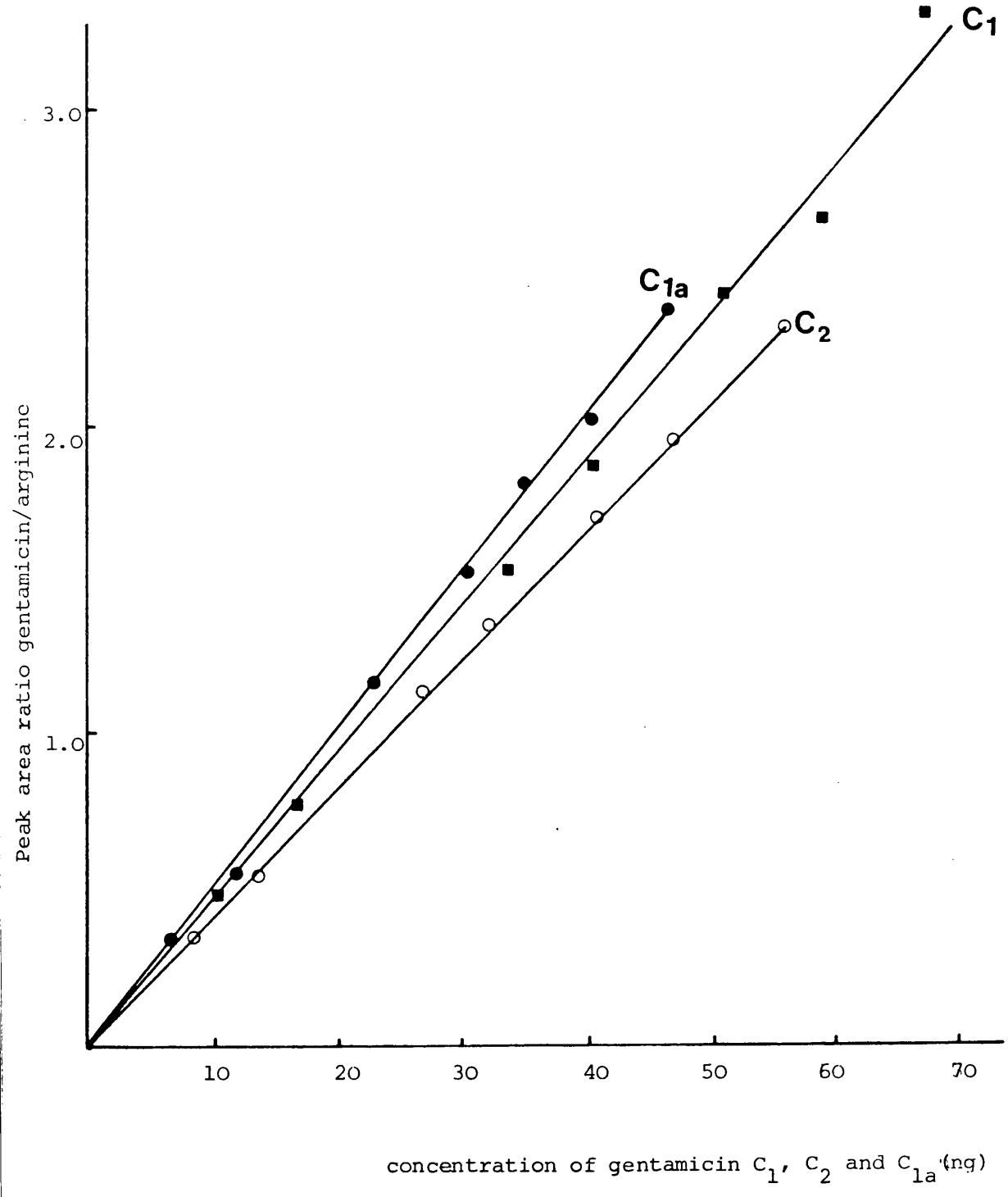
Figure 74. Chromatograms of derivatised neomycin and kanamycin



(2) Calibration graphs:

Figure 75 shows the calibration graphs for the three major gentamicin components. By the use of these graphs the weights of gentamicin $C_{1a}$, $C_2$ and $C_1$ in an unknown can be determined. A series of 21 determinations were performed on a single sample to give coefficients of variation of 2.64, 2.18 and 1.23% for gentamicin $C_{1a}$, $C_2$ and $C_1$ respectively.

Figure 75. Calibration graphs of gentamicin components



3) Assay of raw materials

A series of 19 commercial samples of gentamicin sulphate from various geographical sources, was examined. Where sufficient material was available each was assayed for water content by loss of weight on drying (223) and for sulphate by titration with barium perchlorate (222). The results are shown in Table 19. Where sufficient material was not available a sulphate content of 32% was assumed for the purposes of calculating the gentamicin component contents.

Table 19

| Sample | % (mg/100 mg) Sulphate | Water |
|---|---|---|
| Nicholas | 31.68 | 3.75 |
| SZ-GMC-8-L-6 | 31.41 | 3.65 |
| SZ-GMC-8-L-7 | 31.54 | 3.81 |
| SZ-GMC-8-L-8 | 31.86 | 3.76 |
| SZ-GMC-8-L-9 | 31.58 | 3.16 |
| Pierrel 061 | 32.32 | 3.80 |
| Pierrel 062 | 32.48 | 3.74 |
| Pierrel 064 | 32.96 | 3.69 |
| Pierrel 065 | 32.06 | 4.09 |
| Pierrel 066 | 31.98 | 3.86 |
| Pierrel 067 | 32.83 | 4.05 |
| B/N $^{O}$-GMS-8M-6080 | 33.56 | 4.57 |

Figures 76, 77, 78, 79 and 80 show representative chromatograms of the gentamicin sulphate mixtures from various sources and Table 20 shows the results of the HPLC assay of each sample .

Figure 76. Chromatogram of gentamicin sulphate mixture from China.

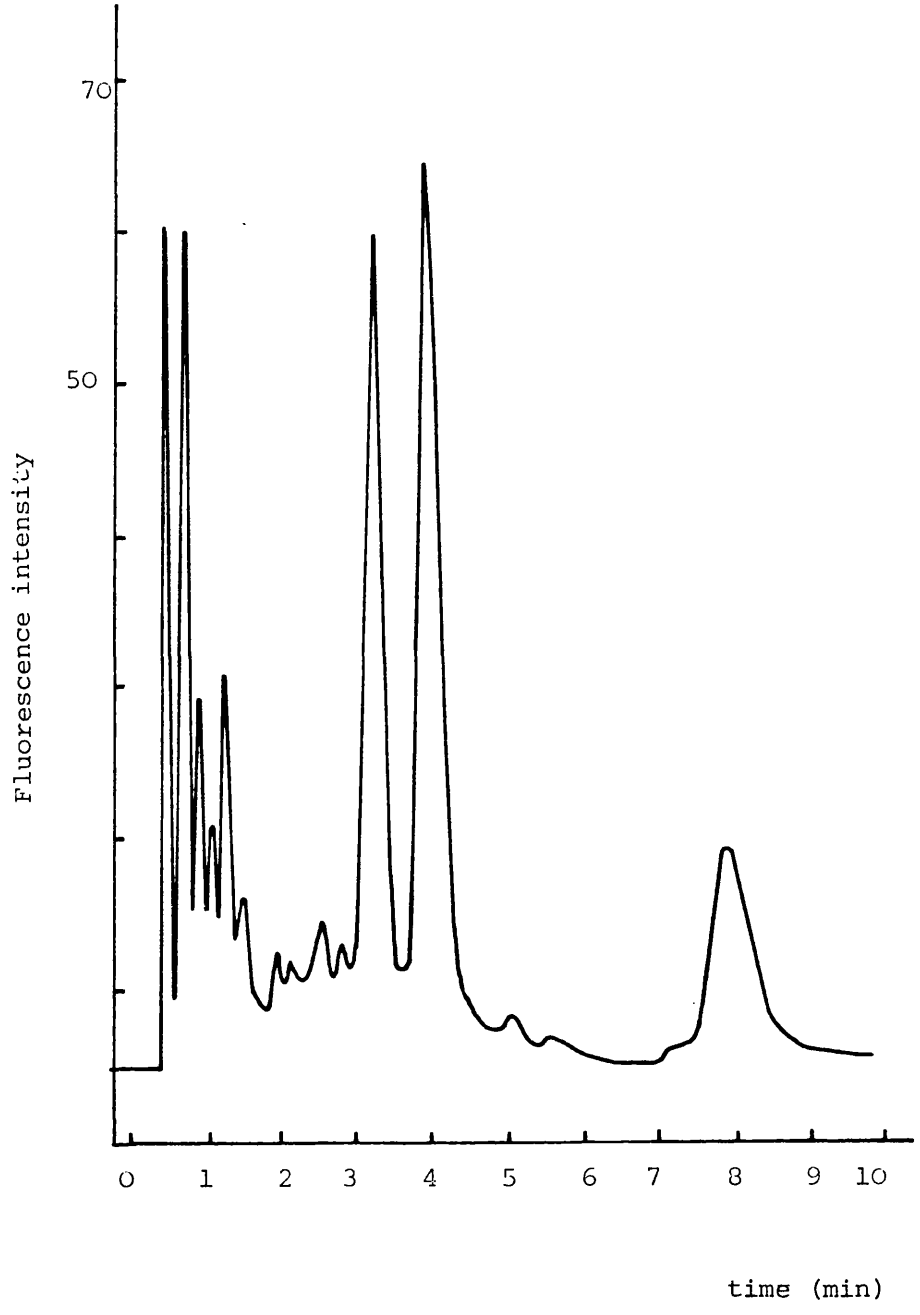


Figure 77. Chromatogram of gentamicin sulphate mixture from Switzerland.

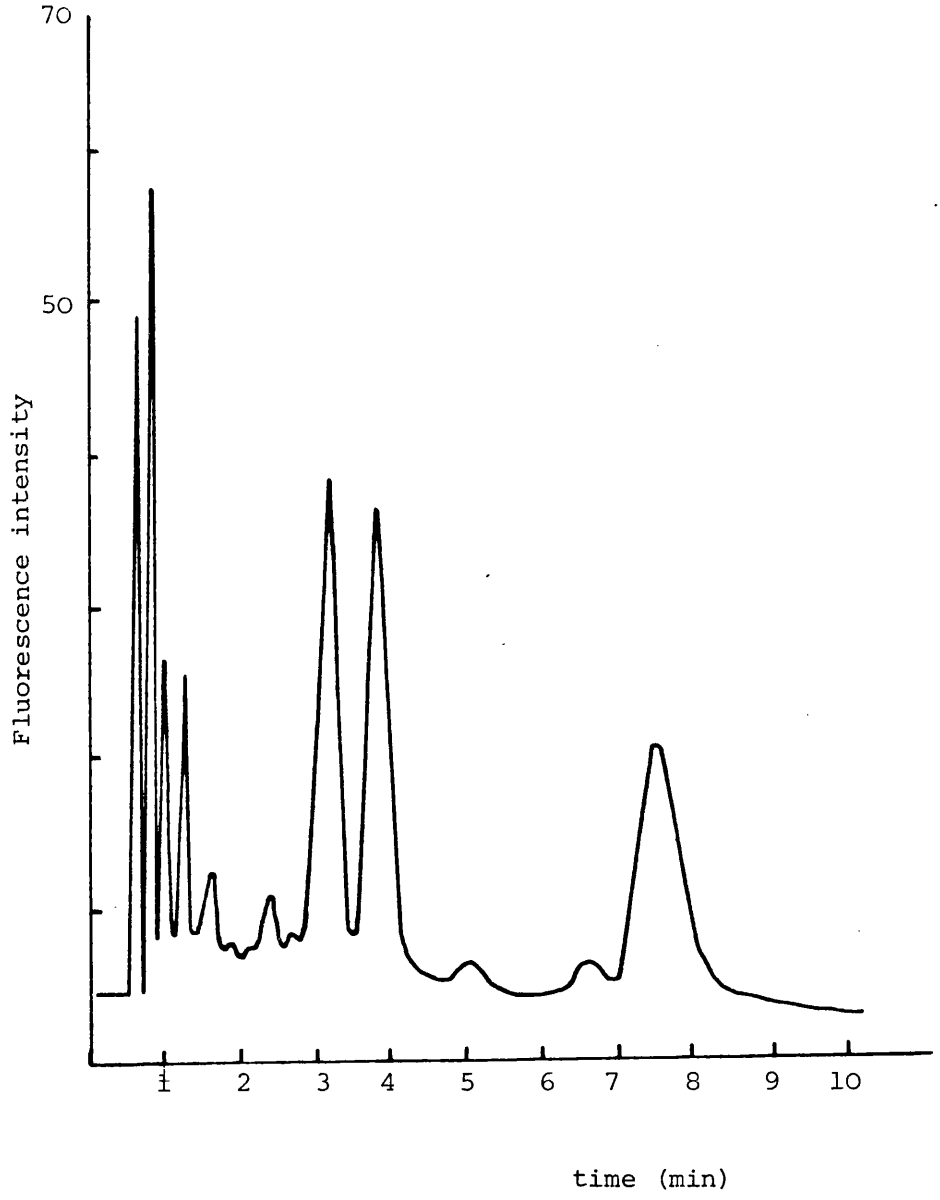


Figure 78. Chromatogram of gentamicin sulphate mixture from Hungary

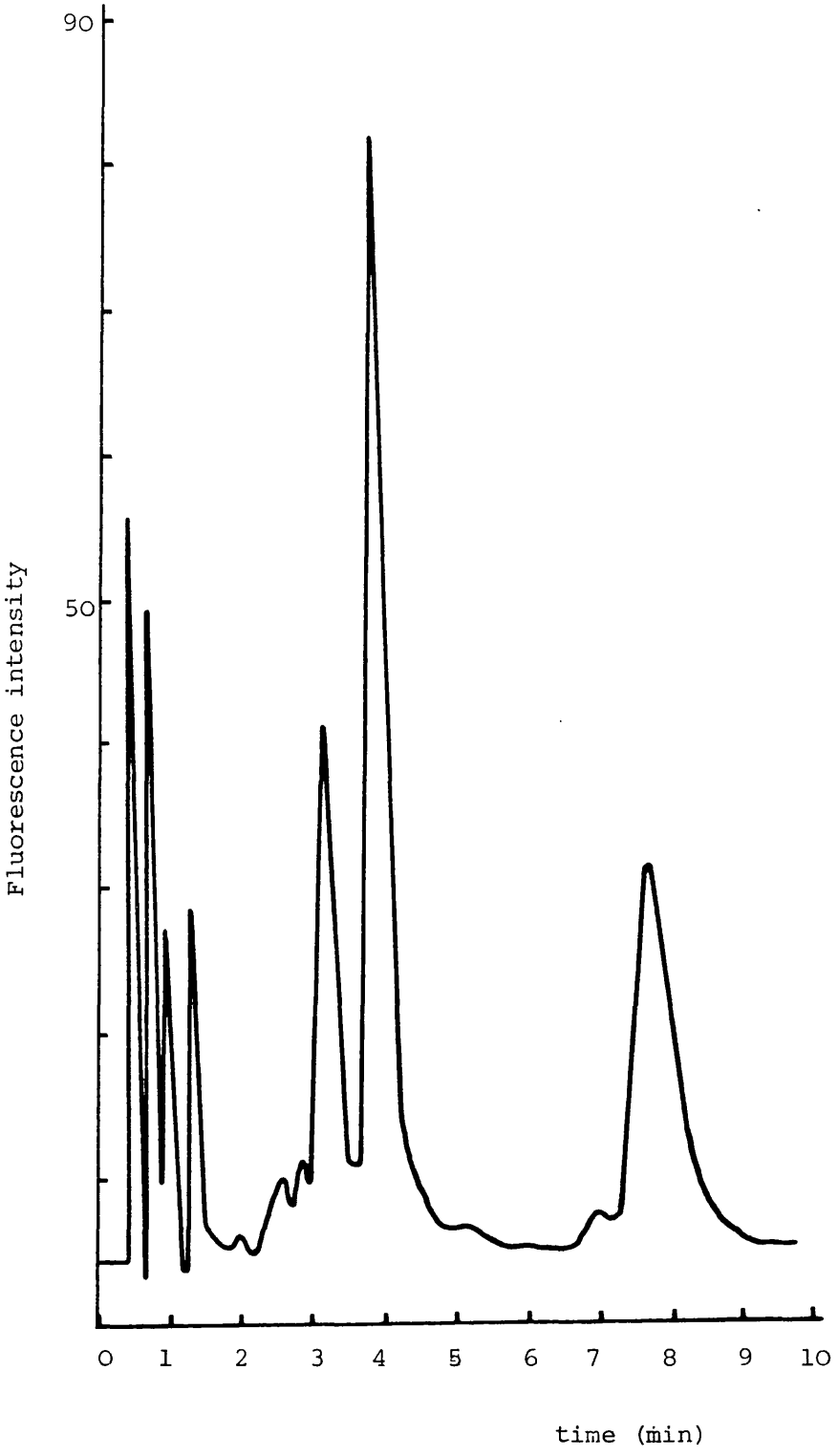


Figure 79. Chromatogram of gentamicin sulphate mixture from Italy

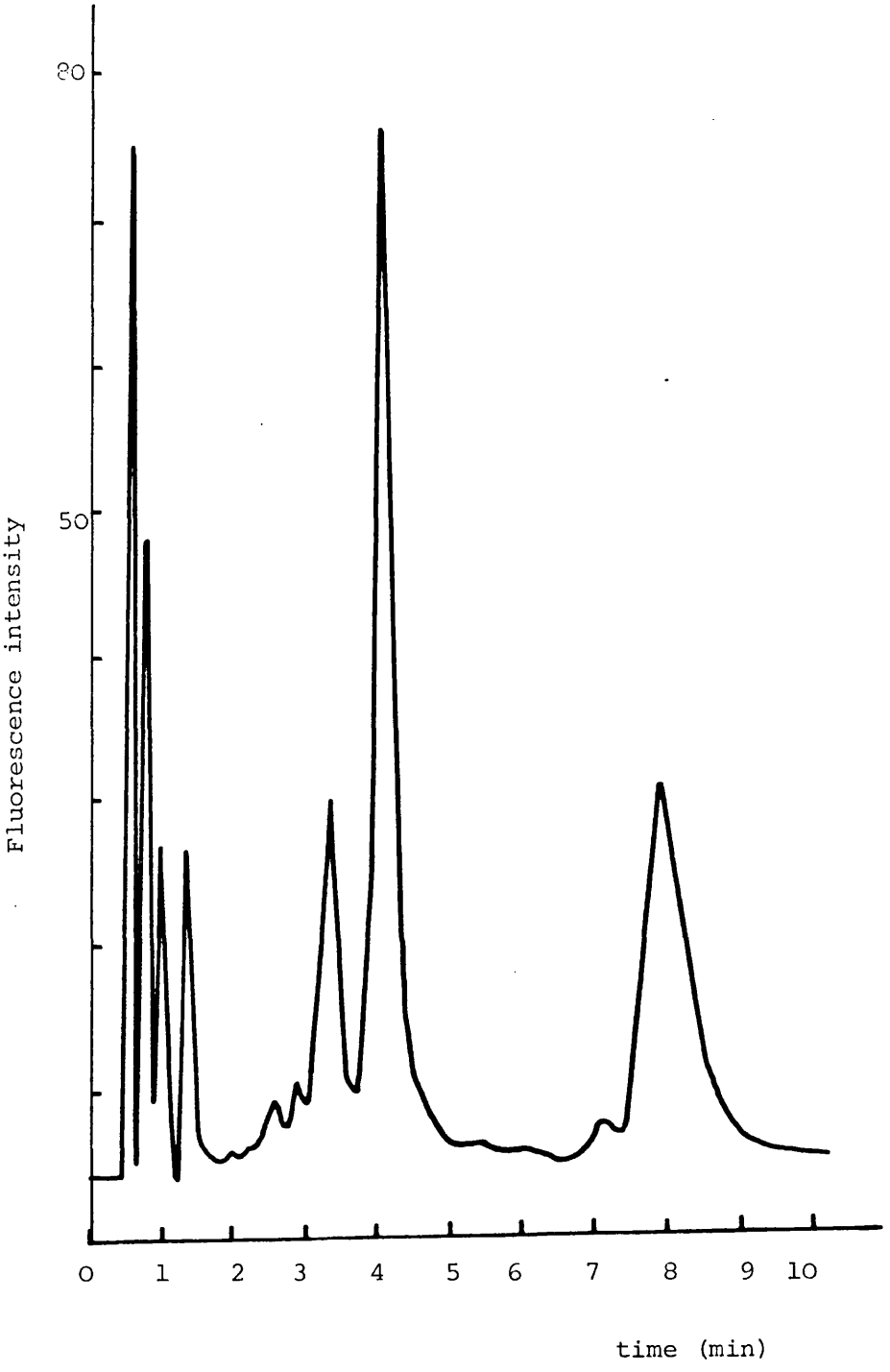


Figure 80. Chromatogram of gentamicin sulphate mixture from Nicholas Laboratories.

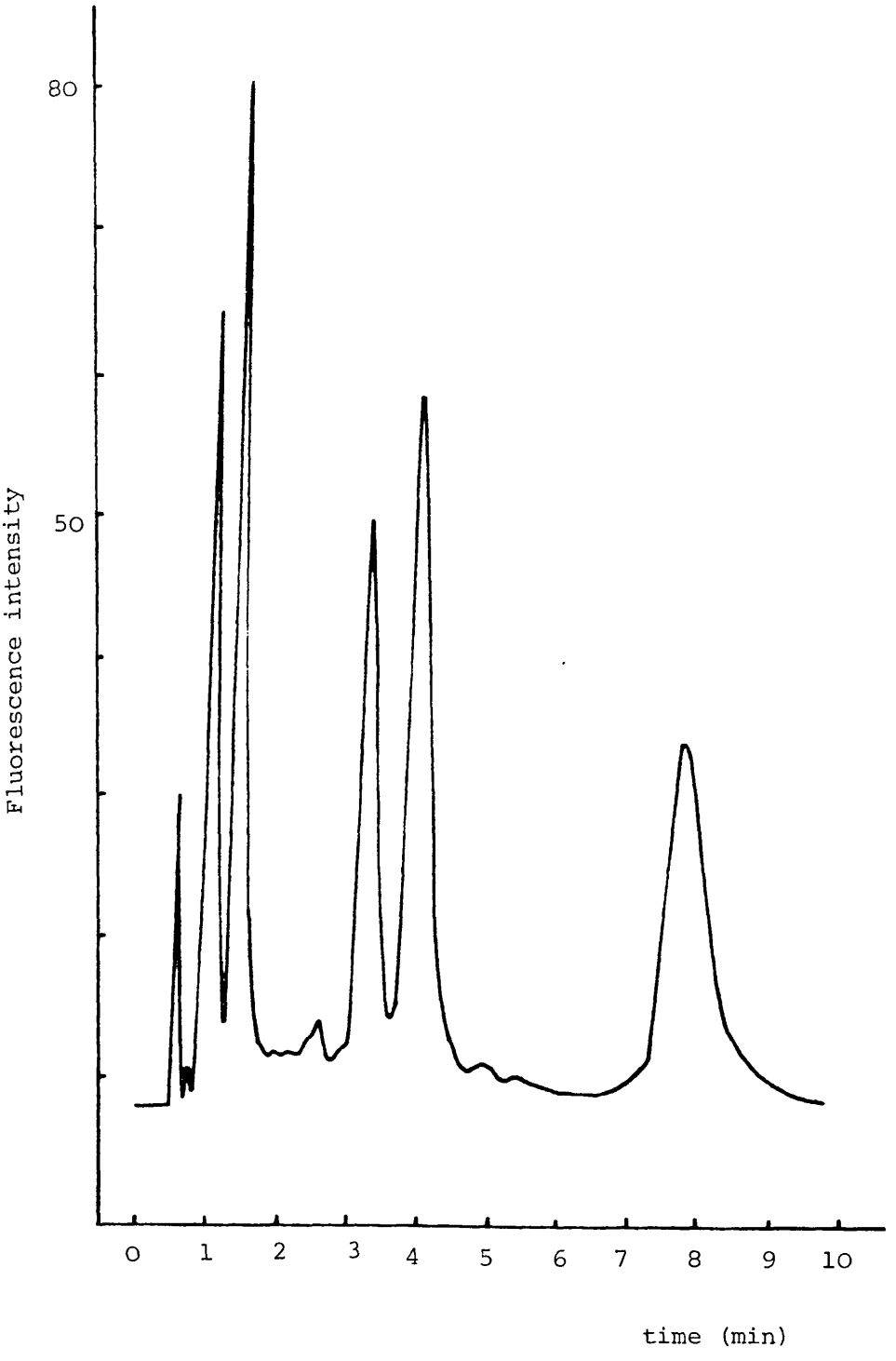


Table 20

| Sample | % (mg/100 mg) water content | $C_{1a}$ | $C_2$ | $C_1$ | total C content | Standard deviation |
|---|---|---|---|---|---|---|
| Nicholas | 3.75 | 23.71 | 37.85 | 35.25 | 96.81 | 4.1 |
| Sample 1 | 3.82 | 23.61 | 35.98 | 14.64 | 74.23 | 2.9 |
| Sample 2 | 3.82 | 12.98 | 41.61 | 39.76 | 94.35 | 0.7 |
| Sample 3 | 3.82 | 15.26 | 45.58 | 37.24 | 98.08 | 1.8 |
| Sample 4 | 3.82 | 19.82 | 50.44 | 32.06 | 102.32 | 2.7 |
| Italian | 3.82 | 15.34 | 49.99 | 32.51 | 97.84 | 3.3 |
| Hungarian | 3.83 | 17.08 | 47.99 | 32.33 | 97.40 | 2.1 |
| Chinese | 3.82 | 22.04 | 39.67 | 20.27 | 81.98 | 4.1 |
| SZ-GMC-8-L-6 | 3.65 | 27.77 | 28.46 | 25.17 | 81.40 | 1.1 |
| SZ-GMC-8-L-7 | 3.81 | 25.38 | 29.27 | 26.54 | 81.19 | 0.6 |
| SZ-GMC-8-L-8 | 3.76 | 24.98 | 24.73 | 24.90 | 74.61 | 1.9 |
| SZ-GMC-8-L-9 | 3.16 | 21.66 | 28.59 | 24.65 | 74.90 | 0.7 |
| Pierrel 061 | 3.80 | 19.09 | 54.67 | 16.22 | 89.98 | 0.8 |
| Pierrel 062 | 3.74 | 19.09 | 54.84 | 15.87 | 89.80 | 1.3 |
| Pierrel 064 | 3.69 | 19.26 | 55.27 | 16.01 | 90.54 | 0.9 |
| Pierrel 065 | 4.09 | 19.16 | 54.60 | 16.06 | 89.82 | 0.9 |
| Pierrel 066 | 3.86 | 19.19 | 54.38 | 16.24 | 89.81 | 1.7 |
| Pierrel 067 | 4.05 | 19.34 | 55.25 | 16.26 | 90.85 | 0.9 |
| B/N°-GMC-8M-6080 | 4.57 | 26.59 | 43.37 | 19.86 | 89.82 | 2.1 |

It is clear that the component profiles of these samples varied more widely than previously reported (164, 151, 163, 160, 161, 107). The geographical origin of the samples seems to be a major source of variability. Thus all seven Italian samples are characterised by a high gentamicin $C_2$ content, as is the single Hungarian sample. The four Swiss samples (Benzian) all contain substantial amounts of gentamicin $C_{2b}$ and another unknown constituent. Material of Chinese origin also has a high content of minor components but a quite different ratio of major components. These differences may well reflect differences in the strain of organism, fermentation conditions, or work up procedure in the different manufacturing plants.

(4) Assay of formulations

Seventeen commercially available gentamicin containing formulations obtained from three different manufacturers were also analysed. Table 21 shows the content of each component and the total expressed as a percentage of the labelled amount.

| Formulation (manufacturer) | % individual component (mg/100 mg) | | | total C content | Standard deviation |
|---|---|---|---|---|---|
| | $C_{1a}$ | $C_2$ | $C_1$ | | |
| Genticin Injectable (Nicholas Lab) | 28.72 | 37.81 | 39.97 | 106.5 | 2.2 |
| Genticin Injectable (Nicholas Lab.) | 28.42 | 37.64 | 34.94 | 101.0 | 1.0 |
| Genticin Intrathecal (Nicholas Lab.) | 25.20 | 36.54 | 36.76 | 98.5 | 4.1 |
| Genticin Paediatric (Nicholas Lab.) | 25.66 | 37.16 | 39.48 | 102.3 | 2.3 |
| Genticin Injectable (Nicholas Lab.) | 28.89 | 32.21 | 38.60 | 99.7 | 1.5 |
| Genticin Eye/ear drop (Nicholas Lab.) | 28.68 | 39.60 | 40.32 | 108.6 | 2.4 |
| Gentisone EC ear drops (Nicholas Lab.) | 33.14 | 34.35 | 20.61 | 97.1 | 1.0 |
| Genticin cream (Nicholas Lab.) | 30.34 | 34.38 | 35.48 | 100.2 | 2.8 |
| Genticin Ointment (Nicholas Lab.) | 29.72 | 38.89 | 35.99 | 104.6 | 3.8 |
| Cidomycin Injectable (Roussel) | 25.74 | 39.86 | 38.10 | 103.7 | 1.9 |
| Garamycin Injection (Warrick) | 27.61 | 37.35 | 32.04 | 97.0 | 3.1 |
| Garamycin Paediatric (Warrick) | 28.80 | 36.95 | 32.55 | 98.3 | 0.6 |
| Garamycin Injection (Warrick) | 29.16 | 38.22 | 34.02 | 101.4 | 0.4 |
| Amps.Cidomycin Inj. (Roussel) | 22.28 | 38.76 | 39.26 | 100.3 | 0.8 |
| Cidomycin Injection Paed. (Roussel) | 28.52 | 36.85 | 33.23 | 98.6 | 2.0 |
| Cidomycin Injection Intrathecal (Roussel) | 27.83 | 39.66 | 44.81 | 112.3 | 0.7 |
| Cidomycin Ointment (Roussel) | 26.43 | 35.82 | 36.55 | 98.8 | 0.3 |

The 'Code of Federal Regulation (1976)' (229) indicates percentage limits for various preparations.

| | |
|---|---|
| gentamicin sulphate injection | 90 - 125% |
| gentamicin sulphate ophthalmic dosage form | 90 - 135% |
| gentamicin sulphate ophthalmic ointment | 90 - 135% |
| gentamicin sulphate cream | 90 - 135% |

It can be seen that all sample formulations assayed by this method fell within official limits.

(5) Assay of gentamicin in plasma

Figure 81 compares the chromatogram for gentamicin in plasma using trichloroacetic acid as protein precipitating agent with those of plasma alone and gentamicin alone.

Table 22 shows peak heights of the gentamicin components when various precipitating agents were used

| Tube number | Peak height (cm) | | | Percentage recovery |
|---|---|---|---|---|
| | $C_{1a}$ | $C_2$ | $C_1$ | |
| 1. Control | 10.60 | 13.80 | 8.15 | control |
| 2. Trichloroacetic acid | 11.45 | 15.50 | 8.25 | 108.1 |
| 3. Ethanol | 4.65 | 6.50 | 4.35 | 47.6 |
| 4. Methanol | 7.20 | 9.45 | 5.45 | 67.9 |

Ammonium sulphate (tube 5) precipitated only a small amount of protein and the supernatant was slightly yellow. After derivatisation and filtration, the solution turned to dark brown and was not suitable for injection.

Figure 81. Chromatogram of gentamicin mixture in plasma.

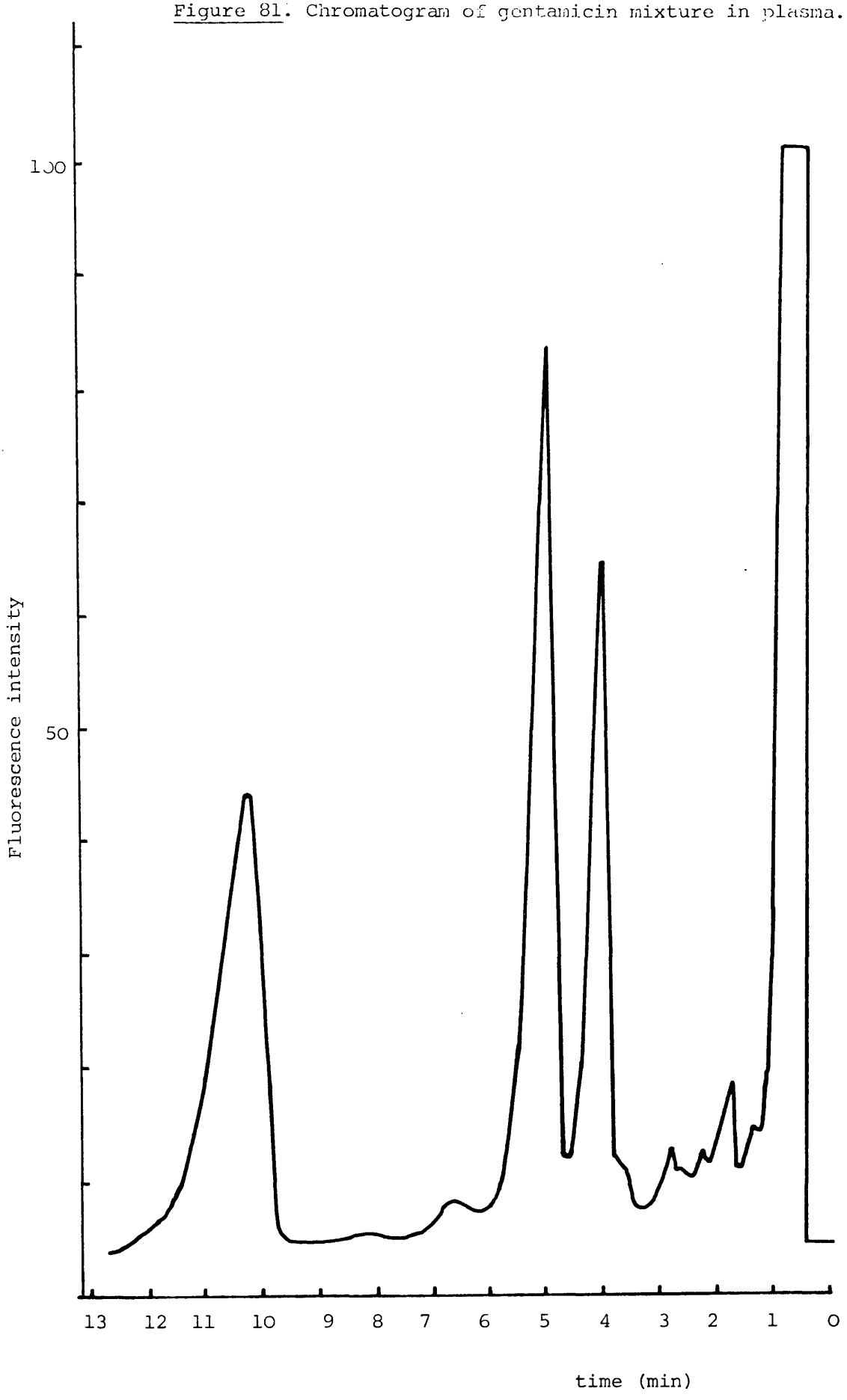


Figure 81. (continued) Chromatogram of gentamicin mixture

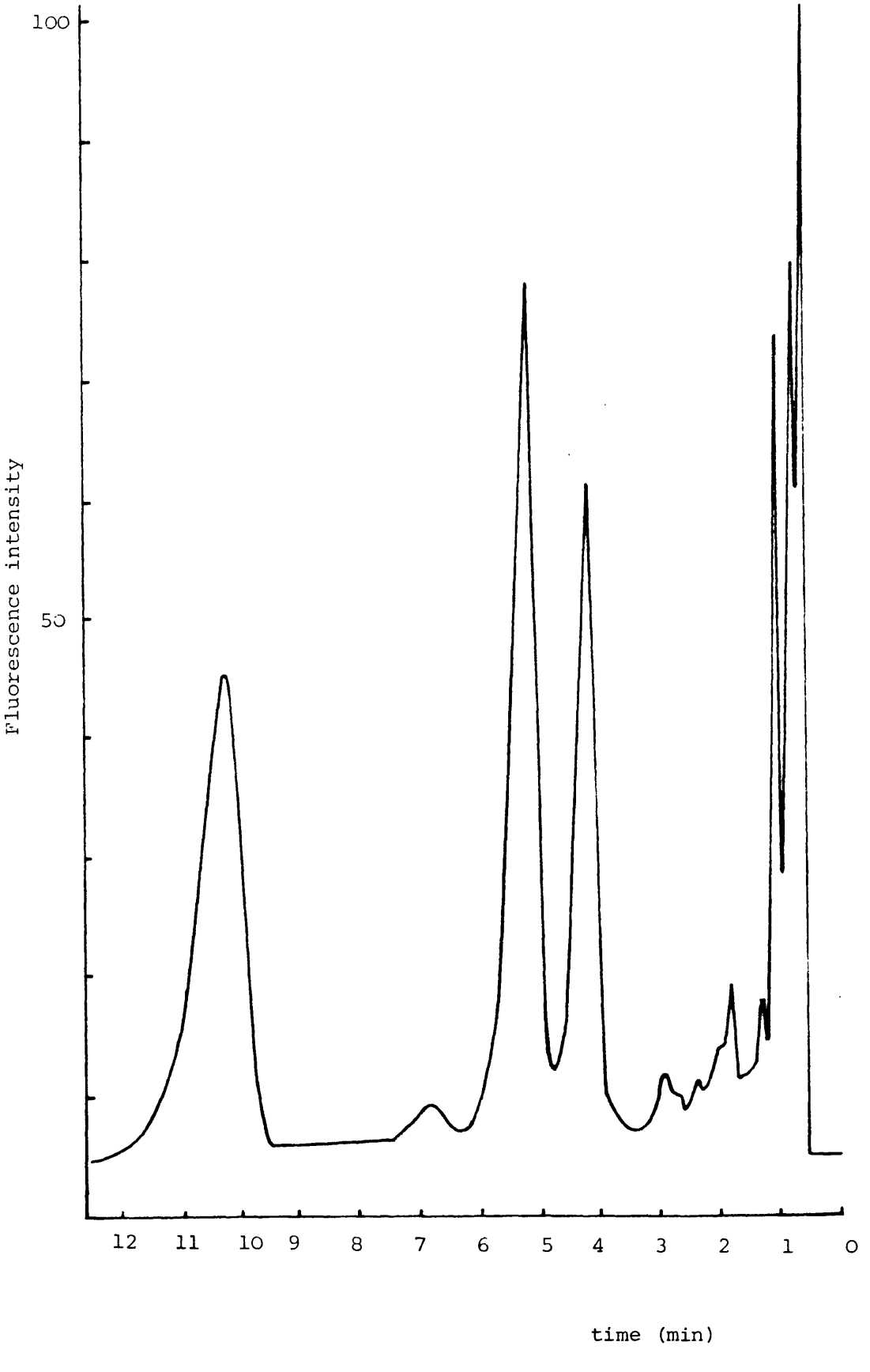
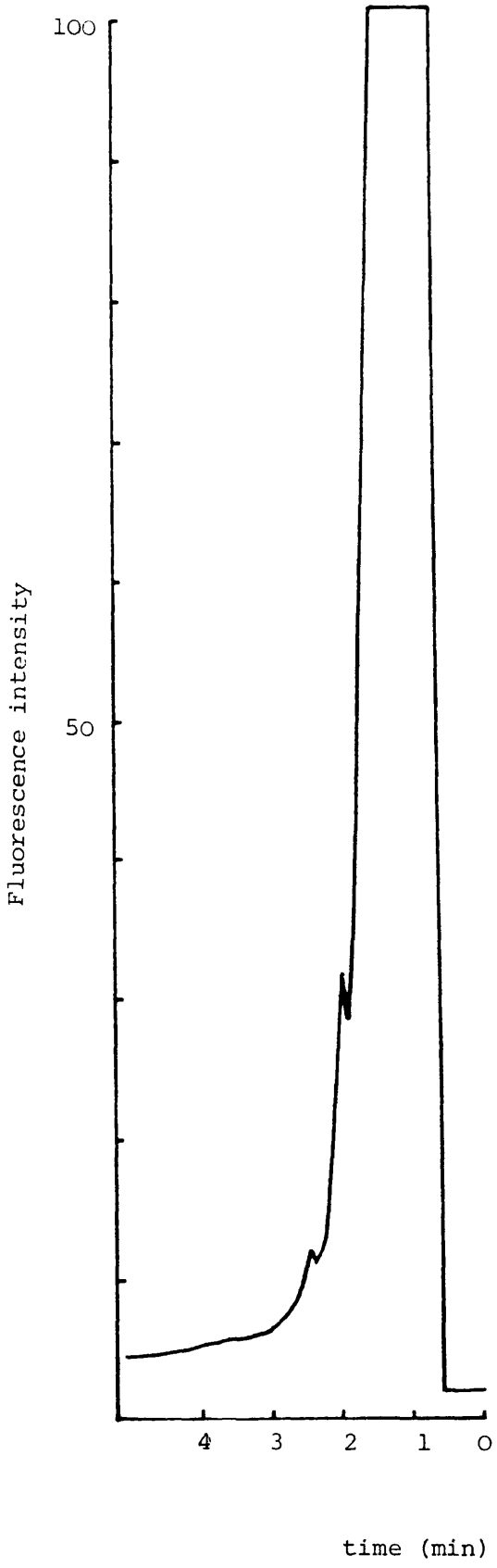


Figure 81. (Continued) Chromatogram of plasma alone



When O-phthalaldehyde was added directly to the plasma (tube 6), the solution remained turbid after centrifugation and filtration and was unsuitable for the injection.

From the above table, it is clear that trichloroacetic acid is the best of the protein precipitating agents examined, giving approximately 100% recovery. However, the broad peak at the start of the chromatogram due to plasma constituents prevents the use of arginine as an internal standard. Quantitation was based on external standardisation with known concentrations of gentamicin.

With ethanol, only half of the drug was detected and with methanol about 70% of the drug was recovered.

Table 23 shows the percentage recovery of gentamicin at various concentrations in the normal therapeutic range when trichloroacetic acid was used as protein precipitant.

| Concentration (µg/ml) | percentage recovery |
|---|---|
| 1 | 107.2 |
| 2 | 94.0 |
| 4 | 95.0 |
| 6 | 98.8 |
| 8 | 106.3 |
| 10 | 97.6 |
| 11 | 97.9 |
| 12 | 103.2 |

| | |
|---|---|
| average percentage recovery | 100.0 |
| coefficient of variation | 4.99% |

Percentage recovery for each component:

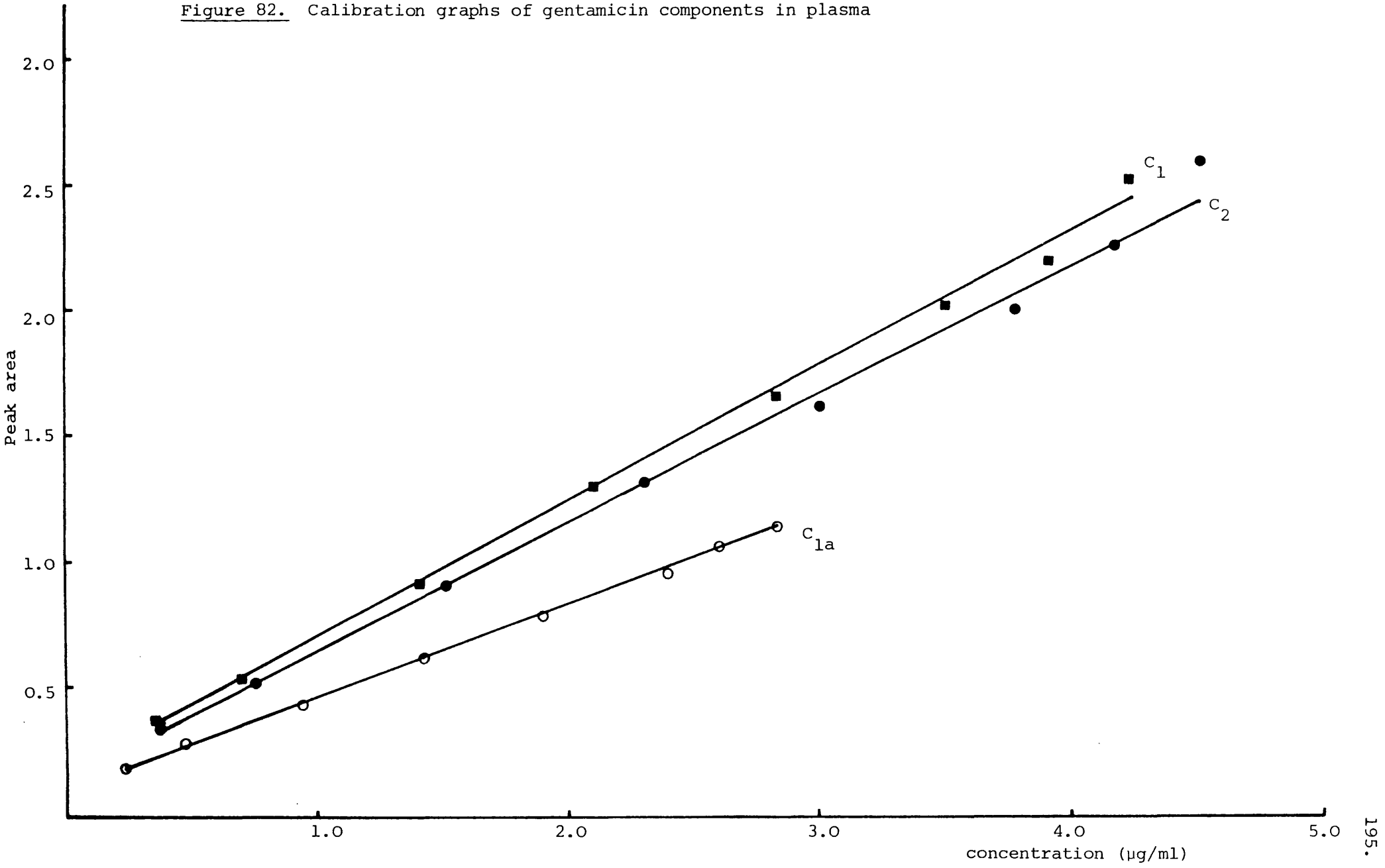
| | |
|---|---|
| gentamicin $C_{1a}$ | 98.4 |
| gentamicin $C_2$ | 99.6 |
| gentamicin $C_1$ | 102.0 |

Maitra et al. (172) who adsorbed gentamicin from serum onto silicic acid and derivatised it in situ reported that with serum concentrations of gentamicin exceeding 2 µg/ml, recovery was between 95 - 105%, but with concentrations near 1.0 µg/ml the recovery was reduced to 80%.

Figure 82 shows the calibration graphs of gentamicin components against concentration which allow the concentration of gentamicin in a plasma sample to be read off. One sample obtained from a patient contained 9.6 µg/ml. This is within the desired therapeutic range. Other drugs (benzyl penicillin, erythromycin and frusemide) present in the plasma sample do not contain primary amino groups and were not derivatised by O-phthalaldehyde. They did not interfere on the chromatogram.

The present work shows that it is feasible to determine gentamicin concentration in plasma using this method. The assay method is rapid, specific and involves no extraction of gentamicin from the plasma.

Figure 82. Calibration graphs of gentamicin components in plasma



# CHAPTER 6

## Proton Magnetic Resonance limit test for gentamicin

### Introduction

The data presented by Calam et al. (154) form the experimental basis for the test intended to control the composition of gentamicin sulphate, and published in the British Pharmacopoeia (1980) (185). This was the first exploitation in the British Pharmacopoeia of nuclear magnetic resonance spectrometry as an analytical technique.

From the NMR spectra of the major components (Figure 83), it is clear that gentamicin $C_1$ contains two N-methyl ($-NH-CH_3$) groups, one secondary C-methyl group ($-CH-CH_3$) and one tertiary C-methyl group ($-\overset{|}{C}-CH_3$). Gentamicin $C_2$ contains one fewer N-methyl groups while gentamicin $C_{1a}$ differs from $C_2$ by the absence of the secondary C-methyl group.

These spectra are of gentamicin bases. The sulphates afford spectra which are a little less clear because one half of the doublet assigned to the secondary C-methyl group is masked by the tertiary C-methyl signal ($\delta$1.36) as illustrated in the spectrum of a commercial mixture of gentamicin sulphate (Figure 84).

The limit test controls the proportion of the major components in commercial gentamicin sulphate mixture by specifying limits for

the ratio of

$$\frac{\delta 1.25 \text{ (secondary C-methyl)}}{\delta 1.35 \text{ (tertiary C-methyl)}}$$ and

for the ratio of $$\frac{\delta 2.75 \text{ (side chain N-methyl)}}{\delta 2.95 \text{ (ring N-methyl)}}$$

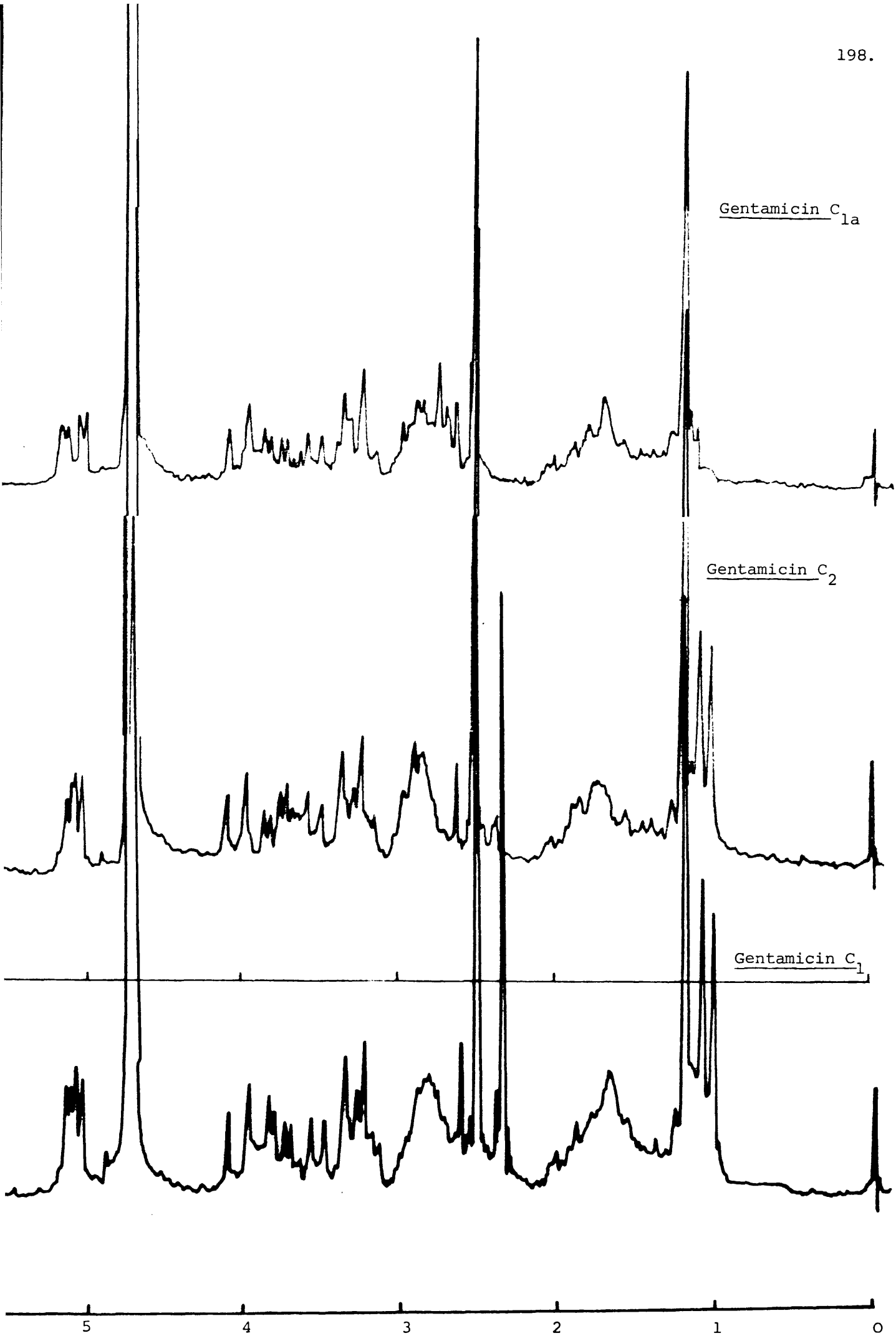
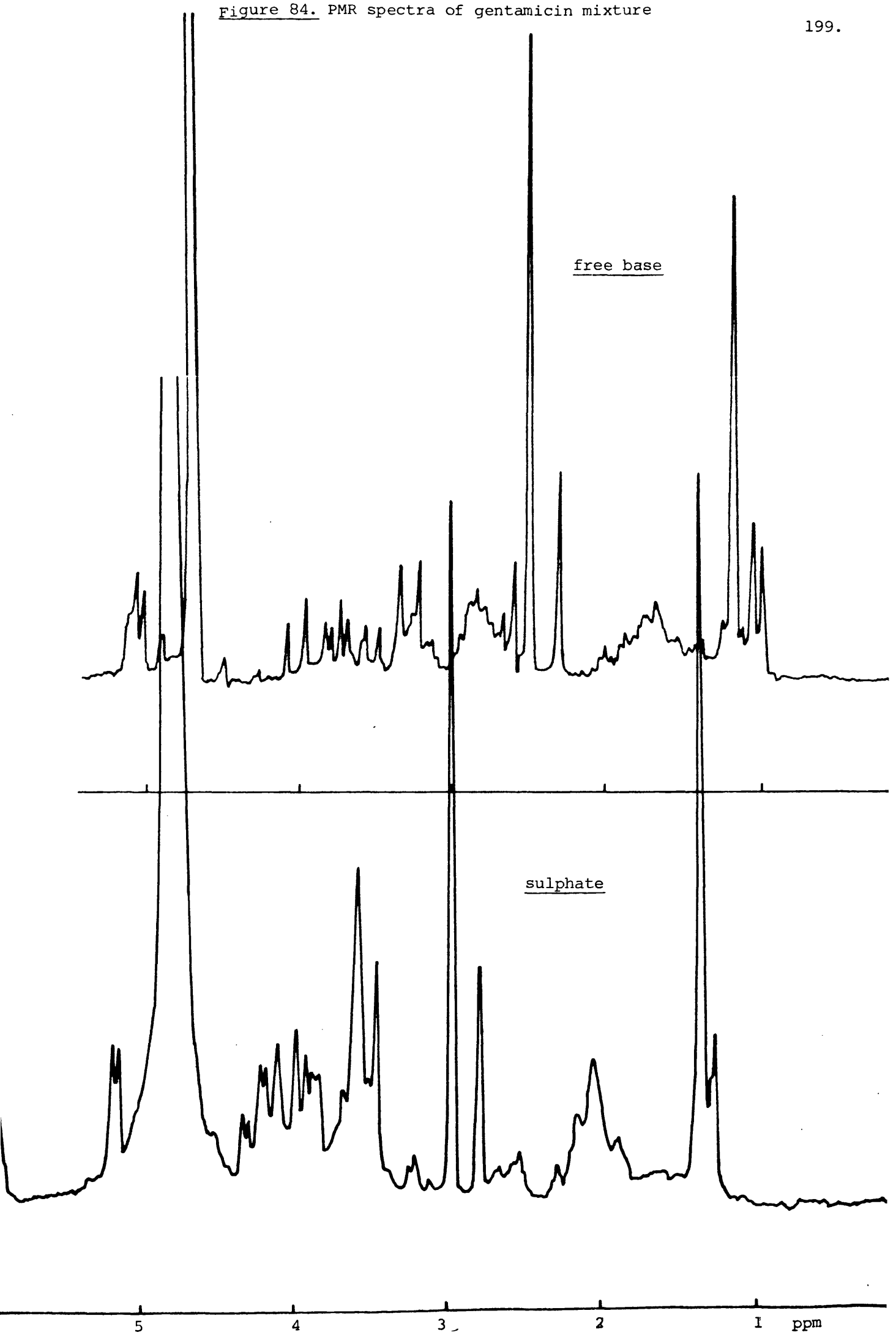



Figure 83. PMR spectrum of gentamicin major components.

Figure 84. PMR spectra of gentamicin mixture



## Materials and Methods

### Materials

Magnesium sulphate was obtained from B.D.H. Chemicals Limited, the reference standard sodium-3-trimethyl silyl propane sulphonate (DSS) from the British Oxygen Company Limited, and deuterium oxide from Aldrich Chemical Company Limited.

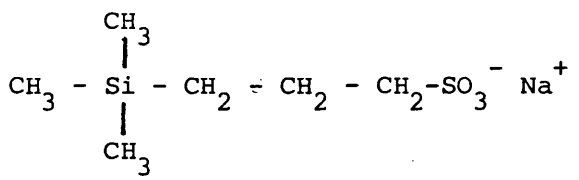
### Instrumental settings

Perkin Elmer R12B 60 MHz NMR Spectrometer

| | |
|---|---|
| Solvent | $D_2O$ |
| Concentration | 20% w/v |
| Reference standard | sodium-3-trimethyl silyl propane sulphonate |
| R.F. Field | 8 |
| Scale | 10 ppm |
| Sensitivity | 4 |
| Filter | 2 |
| Sweep time | 18 |

### Method (B.P. 1980)

The proton absorption spectrum of a 20% w/v solution of gentamicin in deuterium oxide which had been filtered into a NMR table and freed from oxygen by slow bubbling of nitrogen was recorded. The frequency reference standard was 0.5% w/v of sodium-3-trimethyl silyl propane sulphonate (DSS).



The spectrometer controls were adjusted using the peak at about δ4.75 (DHO peak) given by a 10% w/v solution of magnesium sulphate ($MgSO_4.7H_2O$) in deuterium oxide as a reference. The phase control was adjusted until the instrument was operating in the pure absorption mode. The scan speed was set at about 1 Hz per second and the filter setting was adjusted such that the noise level of the baseline between δ0 and δ0.5 was in the range 1 to 3% of full scale deflection. The phase control was further adjusted, if necessary, by reference to the mean level of the base line between δ0.1 and δ0.6 and that between δ6.1 and δ6.6.

The heights of the peaks at about δ2.95 ($NH-CH_3$ in all three components) and about δ1.35 ($C-CH_3$ in all three components were recorded at successive radiofrequency settings. The highest setting at which the height of the peak at about δ2.95 was greater than that at about δ1.35 was used. The sensitivity control was adjusted so that the peak at about δ2.95 was as near full scale deflection as possible.

The heights of the peaks with chemical shifts of approximately δ1.25, δ1.35, δ2.75 and δ2.95 were measured with respect to a baseline drawn between the mean baseline between δ0 and δ0.5 and that between δ6.1 and δ6.6, and the ratios δ1.25/δ1.35 and δ2.75/δ2.95 were calculated. The figures quoted in Table 24 are mean values from five spectra.

Result and Discussion

| Sample | Country of Origin | Peak height ratio δ1.25/δ1.35 | Peak height ratio δ2.75/δ2.95 |
|---|---|---|---|
| Nicholas 61101 | U.S.A. | 0.241 | 0.298 |
| Nicholas 5 x 600 2 | | 0.233 | 0.371 |
| SZ-GMC-8-L-6 | | 0.225 | 0.337 |
| SZ-GMC-8-L-7 | | 0.227 | 0.337 |
| SZ-GMC-8-L-8 | Switzerland | 0.242 | 0.353 |
| SZ-GMC-8-L-9 | | 0.232 | 0.338 |
| Pierrel 061 | | 0.250 | 0.277 |
| Pierrel 062 | | 0.266 | 0.294 |
| Pierrel 064 | | 0.276 | 0.281 |
| Pierrel 065 | Italy | 0.270 | 0.294 |
| Pierrel 066 | | 0.274 | 0.290 |
| Pierrel 067 | | 0.276 | 0.293 |
| Pierrel | | 0.251 | 0.284 |
| B/N°-GMC-8M-6080 | U.S.A. | 0.260 | 0.312 |

The B.P. specifies that the ratio of the height of the peak at about δ2.75 ($NH-CH_3$ in gentamicin $C_1$) to that of the peak at about δ2.95 should be between 0.260 and 0.440, and that the ratio of the height of the peak at about δ1.25 (one peak of the methyl doublet for $CH-CH_3$ in gentamicin $C_1$ and $C_2$) to that of the peak at about δ1.35 should be between 0.200 and 0.260.

All the samples complied with the B.P. specification at ratio δ2.75/δ2.95 but 5 samples did not comply at ratio δ1.25/δ1.35

due to a high content of gentamicin $C_1$ and $C_2$ as indicated by the $CH-CH_3$ signal.

In addition to its use in this limit test the ratio of the N-methyl peak at δ2.75 to that at δ2.95 can be used to estimate the gentamicin $C_1$ content of the sample. Thomas (230) has compared this method with two chromatographic methods. He suggested that because of its speed and simplicity this is the method of choice for checking that a sample of gentamicin complies with a defined composition, especially when used in conjunction with other tests in the pharmacopoeial monograph.

# CHAPTER 7

## General Discussion

Gentamicin sulphate is known to consist of a mixture of three major components all of which possess antimicrobial activity and several minor components, of which the biological activities are not well known.

Current official assays for gentamicin sulphate depend primarily upon a microbiological estimation of total potency combined with other tests to control purity. In the B.P. the ratio of major components is controlled within broad limits by means of an NMR test whilst the U.S.P. uses paper chromatography followed by a bioautographic assay.

In the present work an attempt has been made to develop a single HPLC assay to measure the amount of each major component and thus assess total potency and control purity and component ratios at the same time. Table 25 compares the results of HPLC, biological assay and NMR limit test applied to 19 samples of commercial gentamicin sulphate and Table 26 compares the results of HPLC and biological assay on 6 samples of gentamicin formulation.

Table 25

| Sample | HPLC assay | | | | | Biological | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $C_{1a}$ | $C_2$ | $C_1$ | Total | S.D. | potency | S.D. | δ1.25/1.35 | δ2.75/δ2.95 |
| 1 | 23.71 | 37.85 | 35.25 | 96.8 | 4.2 | 100.0 | | | |
| 2 | 23.61 | 35.98 | 14.64 | 74.2 | 2.9 | 99 0 | 4.2 | 0.233 | 0.371 |
| 3 | 12.98 | 41.61 | 39.76 | 94.4 | 0.76 | 96.1 | 6.6 | - | - |
| 4 | 15.26 | 45.58 | 37.24 | 98.1 | 1.8 | 96.5 | 4.8 | - | - |
| 5 | 19.82 | 50.44 | 32.06 | 102.3 | 2.6 | 100.9 | 4.0 | - | - |
| 6 | 15.34 | 49.99 | 32.55 | 97.8 | 3.3 | 98.1 | 4.2 | - | - |
| 7 | 17.08 | 47.99 | 32.33 | 97.4 | 2.1 | 97.0 | 5.0 | - | - |
| 8 | 22.04 | 39.67 | 20.27 | 82.0 | 4.1 | 95.9 | 5.2 | - | - |
| 9 | 27.77 | 23.46 | 25.17 | 81.4 | 1.1 | 99.7 | 4.4 | 0.225 | 0.337 |
| 10 | 25.38 | 29.27 | 26.54 | 81.2 | 0.6 | 93.6 | 5.1 | 0.227 | 0.337 |
| 11 | 24.98 | 24.73 | 24.90 | 74.6 | 1.9 | 97.6 | 7.8 | 0.242 | 0.353 |
| 12 | 21.66 | 28.59 | 24.65 | 74.9 | 0.7 | 100.6 | 1.3 | 0.232 | 0.338 |
| 13 | 19.09 | 54.67 | 16.22 | 90.0 | 0.8 | 100.5 | 3.8 | 0.250 | 0.277 |
| 14 | 19.09 | 54.84 | 15.87 | 89.8 | 1.3 | 97.7 | 1.9 | 0.266 | 0.294 |
| 15 | 19.26 | 55.27 | 16.01 | 90.5 | 0.9 | 93.3 | 4.7 | 0.276 | 0.281 |
| 16 | 19.16 | 54.60 | 16.06 | 89.8 | 0.9 | 93.6 | 7.2 | 0.070 | 0.294 |
| 17 | 19.19 | 54.38 | 16.24 | 89.9 | 1.7 | 101.8 | 7.02 | 0.274 | 0.290 |
| 18 | 19.37 | 55.05 | 15.26 | 90.9 | 0.9 | 95.1 | 4.2 | 0.276 | 0.293 |
| 19 | 26.59 | 43.37 | 19.86 | 89.8 | 2.1 | 91.7 | 4.6 | 0.260 | 0.312 |

Table 26.

| | Percentage labelled | |
|---|---|---|
| | HPLC | microbiological assay |
| Gentamicin injection | 101.0 | 101.3 |
| Gentamicin paediatric | 100.2 | 101.3 |
| Gentamicin Eye/Ear drops | 108.6 | 104.2 |
| Gentamicin/Hydrocortisone | 97.1 | 102.3 |
| Gentamicin ointment | 104.6 | 98.9 |
| Gentamicin Intrathecal | 98.5 | (a) |

(a) This method is not suitable due to the inhibition of bacterial growth of saline controls.

It is notable that in the latter case agreement between the HPLC method (expressed as the total of major constituents) and the biological method is good whereas it is much less good for the commercial raw materials. In their assay of gentamicin in plasma Maitra et al. (172) found extremely good correlation between their HPLC results and a biological assay.

It is clear that these differences in correlation between the two methods depend on the nature of the sample being analysed. Thus Maitra's assay was carried out on plasma from a human volunteer who had received a single dose of gentamicin intravenously. All the formulations reported in Table 21 contained gentamicin sulphate B.P. and the tables show that these varied much less in their HPLC profiles than the raw material samples.

Table 25 shows that whereas the microbiological activities of the raw material samples do not differ significantly from each other the chemical content of major components does. This implies that one or more of the minor components present does possess biological activity which contributes to the microbiological activity of the mixture. Several workers (107, 158, 104) have drawn a similar conclusion from a disparity between chemical and biological assays and two minor constituents gentamicin $C_{2a}$ and $C_{2b}$ have been isolated (168). However, Thomas and Tappin (151) have isolated 3 minor components by ion exchange chromatography which had no antimicrobial activity. In this work at least 7 minor components have been isolated from a commercial sample of gentamicin (sample 1) and have been shown to possess antimicrobial activity. Some of these may be identical to compounds of Micromonospora purpurea var. nigrescens strain by Bérdy et al. (166). It is interesting that sample 1 is in fact one of the purest of those examined.

The precision of both assays is shown by the values of the standard deviations. Although 30 replicates were performed in the microbiological assay as opposed to 5 in the HPLC assay the minimum standard deviation obtained in the biological assay was 1.3 and with most of the samples the values are higher than that of the HPLC method.

Table 25 also shows the results of the B.P. NMR limit test carried out on commercial samples of gentamicin. Samples 1, 9, 10, 11, 12, 13 and 19 all complied with the B.P. requirement though sample 9 to 12 inclusive (the Swiss samples) all had a relatively high content of minor components. Samples 14 to 18 inclusive

failed to comply due to a high δ1.25/δ1.35 ratio, consistent with their high proportion of gentamicin $C_2$.

Thus the broad limits imposed on the gentamicin C component ratio by the B.P. NMR limit test will detect and exclude large deviations from the 'usual' ratio but seem to be insensitive to the presence of minor components. Studies on the biological properties of gentamicins $C_1$, $C_{1a}$ and $C_2$ suggest that these compounds differ only slightly in both antimicrobial activity and toxicity (12). Thus the observed variations in the major component ratios may be of little therapeutic significance whereas the presence of minor components of unknown biological activity could be far more significant.

It is likely that in the future gentamicin sulphate will become available from a wider variety of manufacturing sources than at present. Current pharmacopoeial standards are based on the restricted sources currently available and it is clear that the natural variation in the new sources will lead to some of them falling outside the present limits. The methods described in this thesis offer a discriminating and flexible means of monitoring the composition of gentamicin and hence of providing data upon which a realistic specification for this valuable antibiotic mixture can be prepared.

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CORRECTIONS

| Page | Line | Correction |
|---|---|---|
| v, vi, 36, 39, 40, 114, 115, 116, 119, 148, 155, 156, 166, 193, 194, | | o - phthalaldehyde |
| 1 | 5 | insert antibiotics which are based on oligosaccharide. The after second of |
| 19 | 19 | assaying |
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| 27 | 5 | cell |
| 36 | 8 | high-performance |
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| 47 | 17,24 | isopropanol |
| 110 | 11 | on |